HETEROCYCLIC PYROPHOSPHATE ANALOGUES AS POTENTIAL ANTIVIRAL AGENTS

by

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A thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy at the University of Warwick

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ABBREVIATIONS

CIMS	Chemical ionisation mass spectrum
DMEM	Dulbecco's modification of Eagle's medium
DNase	Deoxyribonuclease
EMEM	Eagle's minimal essential medium
H	Haemagglutinin
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
HSV-1, -2	Herpes Simplex Virus, type 1, type 2
ID_{50}	50% inhibitory dose
M	Matrix
N	Neuraminidase
NEAA	Non-essential amino acids
NOE	Nuclear Overhauser effect
NP	Nucleoprotein
PAA	Phosphonoacetic acid
PFA	Phosphonoformic acid
p.f.u.	plaque forming units
$pK_{d'}$	$-\log_{10}$ (dissociation constant for complex formed between zinc ions and pyrophosphate analogue at pH 8.0)
t.l.c.	thin layer chromatography

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DECLARATION

The work described in this thesis is the original work of the author except where acknowledgement is made to work and ideas previously published. It was carried out in the Department of Chemistry, University of Warwick and at the Department of Applied Biology, Roche Products Ltd., Welwyn Garden City, between October 1982 and September 1985, and has not been submitted previously for a degree at any institution.

M. Naylor

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Above all, I would like to thank Dr. D. W. Hutchinson for his unwavering enthusiasm, encouragement and a relentless source of ideas throughout the course of this project.

PUBLICATIONS

Parts of the research described in this thesis have appeared in the scientific literature as follows:

1. Thio-analogues of inorganic pyrophosphate inhibit the replication of influenza virus A *in vitro*
Hutchinson, D. W., Naylor, M. and Cullis, P. M. (1985)
Antiviral Res., 5, 67-73

2. Pyrophosphate analogues as inhibitors of viral polymerases
Hutchinson, D. W., Naylor, M., Semple, G., and Cload, P. A. (1985)
Biochem. Soc. Trans., 13, 752-753

3. Inhibition of viral nucleic acid synthesis by analogues of inorganic pyrophosphate
Hutchinson, D. W., Naylor, M. and Semple, G. (1985)
Chemica Scripta, in press

4. The effect of pyrophosphate analogues on the replication of different strains of influenza virus in tissue culture
Hutchinson, D. W. and Naylor, M. (1985)
submitted to *I.R.C.S. Med. Sci.*

5. The antiviral activity of tetrazole phosphonic acids and their analogues
Hutchinson, D. W. and Naylor, M. (1985)
submitted to *Nucleic Acids Res.*

ABSTRACT

A series of novel and known heterocyclic pyrophosphate analogues have been synthesised and screened for activity against herpesvirus DNA polymerases, influenza A RNA transcriptase and calf thymus DNA polymerase α.

A direct correlation between the compounds' abilities to form complexes with zinc ions, as determined by a gel filtration method, and their effectiveness as inhibitors of the RNA transcriptase activity of influenza A virus and in some cases herpesvirus DNA polymerase, has been found. Introduction of 'soft' ligands such as nitrogen and sulphur into pyrophosphate and its analogues has resulted in improved antiviral activities, in particular, replacement of a phosphoryl group with a thiophosphoryl group has resulted in the discovery of some potent inhibitors of influenza A RNA transcriptase activity. ^{31}P n.m.r. measurements have indicated that these compounds bind to zinc through sulphur, which could account for their increased antiviral activity if they bind in the same way to the essential zinc ion in viral polymerases. The most active of the heterocyclic pyrophosphate analogues, 5-(thiophosphonomethyl)-1(H)-tetrazole, showed improved efficacy against both influenza A RNA transcriptase activity and HSV-1 DNA polymerase activity over its non-thio analogue, 5-(phosphonomethyl)-1(H)-tetrazole.

Some nucleoside-5' esters of 5-(phosphonomethyl)-1(H)-tetrazole were synthesised. These compounds were ineffective inhibitors of the RNA transcriptase activity of influenza and the DNA polymerase activity of herpesviruses, they did, however, exhibit some activity in tissue culture systems. These compounds may therefore act via a mechanism other than inactivation of viral polymerases, or be hydrolysed by cellular enzymes to give the free phosphonate.

A strain dependence has been observed for the inhibition of influenza A virus in tissue culture by plaque reduction assay. Influenza A/WS was found to be the most sensitive of a series of strains tested. Some variation was also observed in the inhibition of different herpesvirus DNA polymerases with the compounds studied.

The uptake of radiolabelled phosphonoacetate and 5-(phosphonomethyl)-1(H)-tetrazole into herpesvirus infected cells has been studied, and a virally-mediated increase in cell permeability to the compounds found to occur maximally 6-7 hours after infection. 5-(Phosphonomethyl)-1(H)-tetrazole shows a small increase in uptake over PAA into infected cells.

CHAPTER 1

INTRODUCTION

1.0 THE DISCOVERY OF VIRUSES AND THE PROBLEM OF VIRAL CHEMOTHERAPY

In the early 1890's Ivanovsky, and later Beijerinck reported the existence of submicroscopic infectious particles in the bacteria-free filtrates of the sap of mosaic-diseased tobacco plants, which were capable of inducing disease in healthy plants. It was Beijerinck who named these pathogenic principles as *"contagium vivum fluidum"*, a living infectious liquid which he called a "virus". In 1898 Loeffler and Frosch were able to transmit foot-and-mouth disease to calves by inoculation with bacteria-free filtrates of lymph of animals infected with the disease. Thus, the existence of mammalian viruses was established.

At the same time the principle of selective toxicity of chemotherapeutic agents for their target organisms was beginning to be considered, culminating with Gultman and Ehrlich's first successful human trial of a chemotherapeutic drug, methylene blue, against the intracellular parasite *Plasmodium vivax*. In 1892 Romanovsky demonstrated the selective toxicity of quinine against the same organism.

However, their submicroscopic size and the fact that viruses required the introduction into living

cells for replication created vast problems for early researchers into possible chemotherapy, and not surprisingly work in this area moved towards the less elusive pathogens such as protozoa and bacteria.

The independent nature of bacteria and the existence of unique enzymes and substrates makes anti-bacterial drugs a relatively easy proposition when one considers the obligate intracellular nature of viruses, which only multiply within living cells able to provide the necessary organelles, metabolic pathways and enzymes or cofactors which they lack. The problem of selective antiviral therapy was therefore, in the early years at least, considered by many to be one that would not be solved by the chemist since chemical agents having any effect on virus multiplication would have a deleterious effect on the host cell.

Since the early 1960's however, considerable advances in the field of viral chemotherapy have been made, with the discovery, usually serendipitously, of a number of truly selective antiviral agents. Advances in modern virology at the molecular level has also meant that many virus-specific processes are now known in detail and a number of virus specific enzymes well characterised, as well as other proteins and specific components of viral replication which specific chemically synthesised blocking agents can be targeted against. Examples of such targets are DNA-dependent DNA polymerases of herpesviruses and RNA-dependent RNA polymerases of

myxo- and paramyxoviruses. Other targets are discussed in Section 1.3.

1.1 VIRUS STRUCTURE AND REPLICATION

The viral genome consists of either DNA or RNA, the latter case being unique in that no other organism employs RNA as its genetic material. Viral nucleic acid may be single- or double-stranded, and may be linear or circular. It may be a polycistronic molecule or the molecules may be separate distinct segmented genomes. Single-stranded viral nucleic acid may also be of positive (viral RNA acts as messenger RNA) or negative (complementary RNA) polarity which must be transcribed to give mRNA by a viral transcriptase (Bishop, 1977). The nucleic acid is enclosed and protected within a protein coat (capsid) to hold the viral nucleic acid in the correct conformation with respect to its transcriptase and to repress the transcription of certain "early genes". The capsid consists of a number of identical subunits (capsomeres) usually arranged with helical or icosahedral symmetry.

The host virally-induced immunological response is largely directed at the protein coat, which may be enclosed within an outer lipid membrane containing viral proteins which are often responsible for specific absorption to susceptible cells.

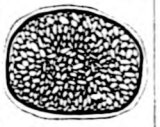
Type of nucleic acid	RNA										DNA				
Capsid symmetry	Icosahedral			Helical							Icosahedral			Complex	
Envelope	Absent		Present								Absent		Present		
Virus family	Picorna	Reo	Toga	Retro	Orthomyxo	Paramyxo	Bunya	Arena	Corona	Rhabdo	Parvo	Papova	Adeno	Unclassified	Herpes	Pox
Morphology																
Size nm	25	70 – 80	40 – 60	100 – 120	80 – 90	120 – 150	90 – 120	90 – 120	80 – 120	50 × 180	20	45 – 55	70 – 80	40 – 45	150 – 200	120 × 270 × 320
Examples of members or group (genus)	Entero (polio, coxsackie, echo) Hepatitis A Rhino	Rota	Yellow fever, RSSE, Rubella	Leukaemia Sarcoma	Influenza A,B,C	Mumps Measles Parainfluenza RS				Rabies		Papilloma Polyoma SV40		Hepatitis B	Herpes simplex Varicella-zoster Cytomegalo Epstein-Barr	Variola Vaccinia Molluscum contagiosum

Fig. 1.1 Summary of the structural properties of different RNA and DNA viruses.
Lycke & Norrby , Textbook of Medical Virology , Butterworth , 1983

The viral replicative process varies with virus type at most stages and since the work described in this thesis is concerned in the main with influenza viruses and herpesviruses, the structure and replication of these viruses will be concentrated upon.

1.1.1 Influenza Viruses

The influenza viruses (orthomyxoviruses) are a large group of viruses, three types of which (A, B, C) are recognised, and all of which cause respiratory tract infections. The first isolation of a virus responsible for human epidemic influenza (Smith, *et al.*, 1933) was the result of years of work on the cause of the 1918-19 influenza pandemic which resulted in 20 million deaths, and has remained the most serious unconquered threat to worldwide human health (Grist, 1979). Epidemics and pandemics arise due to antigenic variation of the haemagglutinin (H) and neuraminidase (N) surface antigens, which can be a result of mutation and immunological selection (antigenic drift) or by replacement of the entire gene coding for the H or N with that of another strain (antigenic shift). Antigenic drift is associated with new epidemics, and antigenic shift can result in a pandemic.

Genetic reassortment is possible because of the unusual virus genome, which is in eight distinct single-stranded RNA pieces. It is thought that this leads to reassortment between human and other strains

of influenza, particularly those infecting birds, resulting in "hybrid" viruses showing antigenic shift (Webster, *et al.*, 1982) and causing pandemics as a result of new surface N and H antigens to which populations have no immunity.

1.1.2 The Influenza Virion

The influenza virus exhibits particles of varying size and morphology but are in general spherical or filamentous (diameter 100 nm) and surrounded by a host cell-derived outer lipid membrane into which the two glycoprotein surface antigens are inserted. The haemagglutinin (H)* occurs as three-sided (14 x 4 nm) rods, and the neuraminidase (N) as four mushroom-type spheres on a rod (Varghese, *et al.*, 1983).

The influenza genome RNA segments are closely associated with a basic nucleoprotein (NP), the antigenicity of which is used to define the three types of influenza (A, B and C). The ribonucleoprotein is surrounded by a shell of hydrophobic matrix protein, within which, and associated in some way with the genome RNA, are three high molecular weight P proteins (PA, PB_1, PB_2) which form the RNA-dependent RNA polymerase complex, which transcribes complementary mRNA molecules from the genome RNA template.

The influenza virus genome consists of eight single-stranded RNA molecules of negative polarity (i.e., viral mRNA is complementary to the genome or virion

* Wilson et al , 1981

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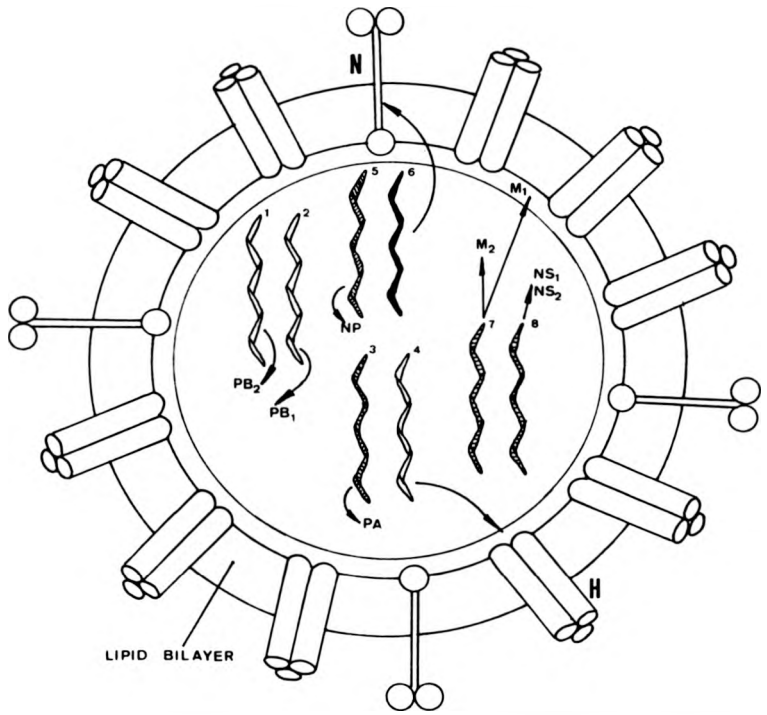

Fig. 1.1.2 The structure of the influenza virion. (not to scale)

RNA (vRNA)). Each segment then acts as a monocistronic message for a virus polypeptide (McGeoch, *et al.*, 1976).

1.1.3 Influenza Virus Replication

Influenza viruses are known to bind to cells by interacting with membrane receptors containing sialic acid via the viral haemagglutinin on the viral membrane. Changes in receptor binding specificity as a consequence of amino acid substitutions in viral haemagglutinins

Table 1-13 Lamb and Choppin, 1983

Influenza virus genome RNA segments and coding assignments

Segment	Length (nucleotides)	mRNA length (nucleotides)	Encoded polypeptide	Nascent polypeptide length (aa)	Approx. no. molecules per virion	Remarks
1	2,341	2,320	PB2	759	30–60	Host-cell RNA cap binding: component of RNA transcriptase
2	2,341	2,320	PB1	757	30–60	Initiation of transcription: possibly endonuclease activity: component of RNA transcriptase
3	2,233	2,211	PA	716	30–60	Elongation of mRNA chains?: component of RNA transcriptase
4	1,778	1,757	HA	566	500	Surface glycoprotein, trimer; major antigenic determinant
5	1,565	1,540	NP	498	1,000	Associated with RNA segments to form ribonucleoprotein: structural component of RNA transcriptase
6	1,413	1,392	NA	454	100	Surface glycoprotein: neuraminidase activity. Tetramer
7	1,027	1,005	M_1	252	3,000	Major protein component of virus: underlies lipid bilayer
		316	M_2	96		Spliced mRNA, nonstructural protein: function unknown
		276	?	?9		Spliced mRNA, peptide predicted by nucleotide sequence only
8	890	868	NS_1	230		Nonstructural protein: function unknown
		395	NS_2	121		Spliced mRNA, nonstructural protein: function unknown
Total	13,588					

suggest that the sialic acid binding site is a surface pocket at the distal end of the H molecule (Wilson, *et al.*, 1981). Further analysis of H structures and their binding specificities and of H-sialyllactose complexes, could lead to specific inhibition of virus adsorption (Skehel and Wiley, 1983).

The next step in the replicative cycle is membrane fusion and penetration. Binding of virus particles to their receptors is followed by endocytosis and fusion of the viral and endosomal membranes. It

appears that haemagglutinin is involved and that the pH optimum of 5.0 (the pH of endosomes) is important in that the H structure is modified at this pH, thus enabling it to interact with the endosomal membranes (Brand, *et al.*, 1980; Huang, *et al.*, 1981).

Changes in endosomal pH may occur on treatment with basic compounds such as amantadine (see Section 1.3.2.1) or by inhibiting the pH maintaining H^+ pump (Galloway, *et al.*, 1983).

1.1.4 RNA Synthesis

After viral entry into the cell and its uncoating, the transcriptase complex must carry out two functions. The eight negatively-sensed monocistronic segments of vRNA must be transcribed into positively-polarised early virus mRNAs required for viral replication, and also complementary full-length transcripts must be produced to serve as templates for the replication of the virus genome RNAs. Because viral mRNAs contain host derived sequences at their 5'-ends and lack sequences complementary to the last 17-22 nucleotides at the 5'-ends of vRNA segments, the viral mRNAs are not suitable templates for replication.

The formation of virus mRNA, mediated by the viral transcription complex (PA, PB_1, PB_2, NP, MP and the eight RNA molecules) is thought to occur in three discrete steps (Lamb and Choppin, 1983; McCauley and Mahy, 1983) as shown in Fig. 1.1.4.

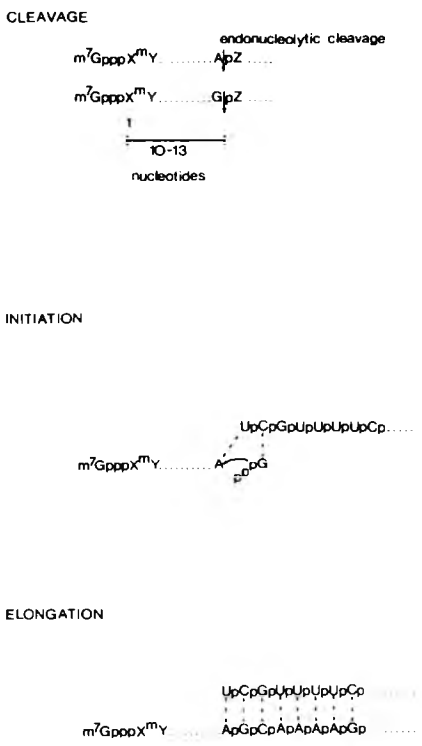

Fig. 1.1.4 Priming and synthesis of influenzal viral mRNA (from Krug, 1983).

(i) A virally-coded endonuclease cleaves from cellular mRNA a capped oligonucleotide which serves as a primer for initiation. It consists of 10-13 nucleotides in the form of a cleaved 5'-terminal fragment from newly synthesised capped (m^7GpppNm-containing) cellular RNAs in the nucleus.

(ii) Initiation of the mRNA synthesis then occurs by the incorporation of a GMP residue complementary to the second base at the 3'end of the viral genome.

(iii) In the elongation reaction it has been shown that the two basic viral proteins PB_1 and PB_2 are required for the cleavage of the host cell mRNA and incorporation of the first nucleotide (Plotch, *et al.*, 1981; Ulmanen, *et al.*, 1983; Blaas, *et al.*, 1982). The PB_1 protein is also implicated in elongation (Braam, *et al.*, 1983; Romanos and Hay, 1984). A highly conserved uridine-free sequence occurs in all influenza mRNA after the host cell-derived sequences (Skehel and Hay, 1978) followed by message-specific sequences.

Viral mRNA contains 3'-terminal poly(A) sequences. Experiments in which hybrids between vRNA segments and poly(A)-containing viral mRNAs were digested with single-strand specific nucleases indicate that the viral mRNAs lack the sequence complementary to the 5'-terminal 20-30 nucleotides of the vRNAs (Plotch and Krug, 1978; Hay, *et al.*, 1977).

Regulation of mRNA synthesis during infection has been studied in chick embryo fibroblasts (Hay, *et al.*, 1977) (Table 1.1.4).

The relative rates of synthesis of the various viral mRNAs at different times of infection correlates closely with the relative rates of synthesis of the proteins encoded by these mRNAs (Hay, *et al.*, 1977; Inglis and Mahy, 1979). This indicates a regulation

Similar amounts of all 8 viral mRNAs	2 mRNAs predominate (i) NP, (ii) NS1 (non-structural protein)	NS1 mRNA synthesis decreases relative to NP mRNA. The rate of mRNA synthesis for M(membrane protein), N, and H increases greatly.

INFECTION → EARLY PHASE → LATE PHASE

Similar low levels of the 3 P proteins throughout infection →

Table 1.1.4 Regulation of mRNA synthesis during influenza virus infection.

of viral gene expression largely at the transcriptional level. The virus-specific products synthesised after infection have been implicated in the regulation of viral mRNA synthesis during infection, but the mechanism of this regulation is unknown. It has been suggested that the NS protein plays a role, since in temperature-sensitive mutants defective in the NS genome, the shift from early to late viral protein synthesis (and hence viral mRNA synthesis) is inhibited in infected cells at the nonpermissive temperature (Koennecke, *et al.*, 1981; Wolstenholme, *et al.*, 1980).

Full-length transcripts of vRNA segments without host primer sequences probably act as templates for vRNA replication. Indeed, full-length transcripts are not associated with polyribosomes but are in the form of nucleocapsids (Hay, *et al.*, 1977). The full-length RNA transcripts are also able to form duplex "panhandle" structures by virtue of the presence of the complements of the vRNA 5'-ends. These structures could serve as recognition signals to form nucleocapsids and be copied by the polymerase P proteins (Krug, 1983).

Little is known about the replicative synthesis of vRNA although it has been suggested that since equimolar amounts of the full-length transcripts are made throughout infection, whereas the vRNAs can vary in rate of synthesis (the three P vRNAs in particular), then copying of various templates probably occurs at various specified times after infection (Hay, *et al.*, 1977).

Following viral polypeptide synthesis on cellular polysomes, the newly synthesised polypeptides are transfered across the rough and smooth endoplasmic reticula where the haemagglutinin and neuraminidase are glycosylated prior to insertion into the cell membrane. At the plasma membrane both glycoproteins are involved in the formation of infectious virus particles. The haemagglutinin precursor glycoprotein is cleaved to form the HA_1 and HA_2 components which are linked by a disulphide bond (Waterfield, *et al.*, 1981). The neuraminidase has a specific role in the formation and release of the virus particles from the infected cell, in which sialic acid residues are removed from viral and cellular glycoproteins and glycolipids, thus removing H receptors from the cell membrane and allowing release of virus particles. This step is a possible target for antiviral drugs since the three dimensional structure of the neuraminidase enzyme has been determined and knowledge of the active site is accumulating (Varghese, *et al.*, 1983; Colman, *et al.*, 1983).

1.1.5 Herpesviruses

The herpesviruses are large icosahedral enveloped DNA viruses noted for their tendency to establish persistent and latent infections and to be reactivated by immunosuppression. There are four human herpesviruses (1) Herpes Simplex (HSV); type 1

causing primary pharyngitis and recurrent "cold sores", also kerato-conjunctivitis and meningo-encephalitis; type 2 causing herpes genitalis and neonatal herpes, (2) Varicella-zoster (VZV); causing primary chickenpox and recurrent herpes zoster ("shingles"), (3) Cytomegalo-virus (CMV); which can cause severe congenital abnormalities in the newborn, lethal pneumonitis and hepatitis in the immunocompromised, (4) Epstein-Barr virus (EBV); causing infectious mononucleosis (glandular fever) in adolescents and persists in B lymphocytes. In addition, there are over eighty viruses which infect vertebrates and invertebrates, many of which are species specific.

The HSV-1 and HSV-2 genomes are linear double-stranded molecules of about 100 x 10^6 molecular weight (Kieff, *et al.*, 1971), and have several characteristic features, (i) terminal repetitions that allow circular genomes to form, (ii) discontinuities in the sugar-phosphate backbone of each strand of the duplex DNA and (iii) internal repeated sequences (Roizman, 1979).

About 50 HSV-infected cell polypeptides (ICPs) have so far been identified, with molecular weights ranging from 12 000 to 220 000 (Honess and Roizman, 1973; Powell and Courtney, 1975). Three groups have been classed as α(immediate-early), β(early) and γ(late). The α polypeptides reach maximal levels 2-4 hours after infection and are subsequently phosphorylated and transported to the infected cell nucleus (Wilcox, *et al.*, 1980). β Polypeptides are synthesised maximally

from 5-7 hours post-infection and include enzymes involved in viral DNA synthesis such as DNA polymerase and thymidine kinase. β Polypeptides require functional α polypeptides via activation of β gene transcription. At least one functional β polypeptide is then required to terminate the synthesis of α polypeptides and activate γ gene expression (12-17 hours after infection) to produce γ virion structural polypeptides (Honess and Roizman, 1974).

Host cell DNA and protein synthesis are shut off during infection at the same time as the synthesis of virus-specific components involved in progeny virus synthesis. Once viral DNA and late protein synthesis are in progress the assembly of infectious progeny virus is initiated.

The completed HSV virion contains the DNA and numerous structural polypeptides, the genome being wound on a protein structure comprised of basic histones and located in the icosahedral nucleocapsid of diameter 100 nm, composed of 162 capsomeres (Wildy, *et al.*, 1960). Also contained in the virion are glycosylated proteins, lipids, polyamines and various enzyme activities. The enveloping lipid skeleton is derived from the infected cell inner nuclear membrane by budding (Roizman and Furlong, 1974). The viral surface enzyme glycoproteins (gA-gF and gγ) are involved in virus attachment, and penetration (gβ), as well as the immune response to infection (gC).

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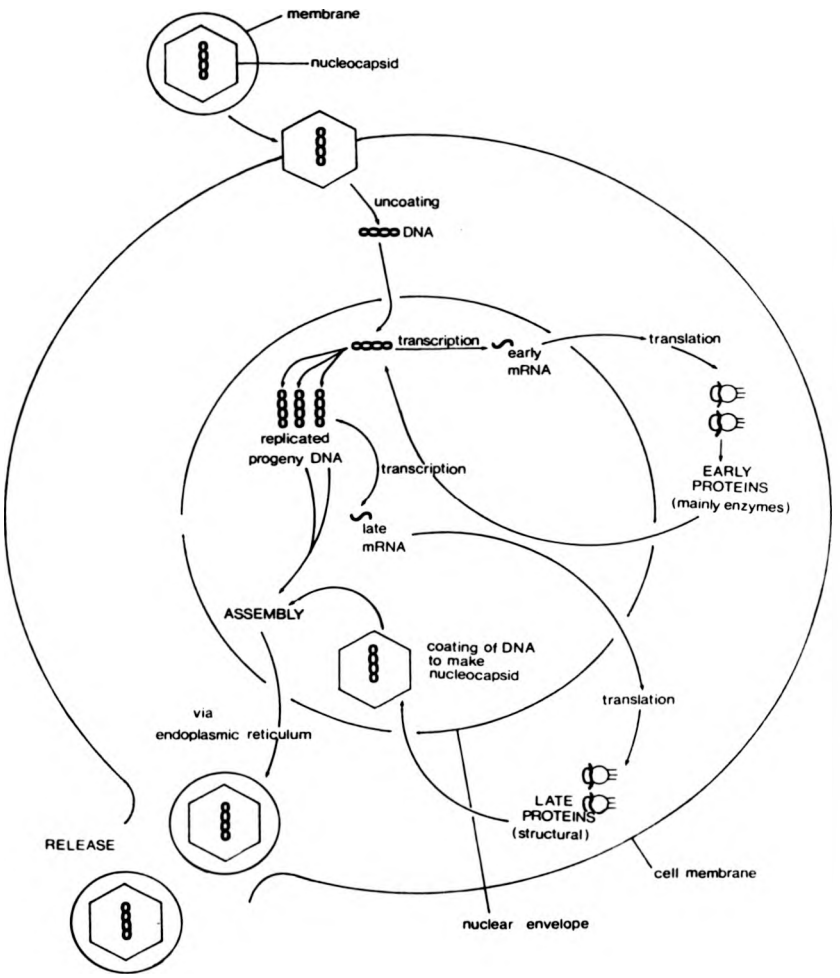

Fig. 1.1.5 Schematic representation of HSV replication in a permissive cell.

The productive replicative cycle eventually leads to cell death, however, the herpesviruses can also survive in a noninfectious or latent form in the nervous system and other tissues of asymptomatic individuals. This strategy of infection enables the virus to avoid elimination by host-mediated influences and despite the development of immunity can persist in the host for long periods. The virus may then become reactivated and after lying in a latent state in the dorsal root ganglia (VZV and HSV) or B-lymphocytes (EBV), pass along nerve trunks to the initial site of infection and cause recurrent infections.

1.2 THE CONTROL OF VIRAL INFECTION

Significant advances towards the control of infectious diseases have been made in recent years, from the simplest methods such as sanitation and personal hygiene improvements, to the development of antibiotics, vaccines and antiviral agents. Such progress has resulted in the erradication of smallpox through the use of vaccinia virus immunisation, and the control of such viral diseases as polio, measles, rabies and mumps, through effective vaccines. New problems will always arise however, such as non-A, non-B hepatitis and acquired immuno-deficiency syndrome (AIDS).

Despite such headway made in the control of viral infection, the principal viral diseases of

our time are still largely unconquered - herpes simplex and zoster, influenza virus, hepatitis, cytomegalovirus and rhinoviruses (common cold), although recent progress has been promising in the herpes area.

1.2.1 Stimulation of the Immune Response - Herpesviruses

The added complication of recurrent exacerbations make a herpesvirus vaccine a valuable target. However, a vaccine would only be able to prevent primary infections, and thereby recurrences after latent infection has been established, since even naturally acquired immunity fails to prevent recurrence.

One of the major problems associated with herpesvirus vaccines has been, with the exception of HSV, the fact that the virus remains cell-associated in tissue cultures resulting in only small numbers of virions in the supernatant and thus making large scale inactivated vaccine manufacture very difficult. Live vaccines may be more accessible, however the possible complications in their use must be considered. They may, for example, be able to establish latent infections, which could manifest itself, perhaps harmfully, years after apparently successful trials may have been completed. Surprisingly, attention has been focussed only recently on the control of herpes simplex virus, responsible for the greatest morbidity of the group, and the only human herpesvirus which grows to high titre in tissue culture. The near epidemic proportions of HSV-2 in some areas is in need

of urgent attention, also worth preventing is perinatal herpes and rare but lethal encephalitis. Subunit type vaccines are the most promising (Skinner, *et al.*, 1982) with an acetone-derived subunit vaccine derived from HSV-1 also being active against the type 2 virus.

As well as conventional approaches, biotechnology may provide a route to a vaccine. An immunologically active chimaeric protein containing a glycoprotein from HSV-1 has been produced, the gene coding for the protein is expressed in *Escherichia coli* (Weis, *et al.*, 1983).

The activation of human T-lymphocytes to synthetic peptides of herpes simplex virus glycoprotein D(gD) has recently been reported (De Freitas, *et al.*, 1985). The major viral glycoproteins are, therefore, implicated as HSV-specific antigens and immunostimulants and are good targets for synthetic peptide programmes.

1.2.2 Influenza Viruses

Influenza virus disease illustrates perfectly the immense problems that can arise in the control of viral infection by immunisation. Influenza is the best known virus capable of antigenic shift and drift, although similar properties are exhibited by human rhinoviruses and foot-and-mouth disease virus. This type of behaviour can render a once effective vaccine obsolete.

Vaccines to date have been egg-grown, formalin-

or β-propiolactone-inactivated and incorporate up to 25 μg of the latest known haemagglutinins. Protection has been lower than seventy percent, particularly for the elderly, one major problem being anamnestic recall of antibody against earlier strains as an immune response to vaccination (Hoskins, *et al.*, 1979). Improvement of response by increased dosage necessitates higher costs and increased side-effects.

Recent developments include the possible use of liposomes (or virosomes), constituted from influenzal H and N. DNA copies of the RNA gene for H have now been cloned in mammalian (Simian) cells using SV40 DNA as vector (Gething and Sambrook, 1981; Sveda and Lai, 1981). This gene has also been incorporated into the vaccinia viral genome by recombination resulting in a potentially useful type of vaccine (Panicali, *et al.*, 1983). The actual *de novo* synthesis of antigenic sites has now become possible by virtue of the rapid determination of H sequences of different influenza strains and a knowledge of the important antigenic sites on the molecule (Green, *et al.*, 1982; Jackson, *et al.*, 1982; Müller, *et al.*, 1982). Peptides have been conjugated with tetanus toxoid for immunisation, however the resulting protection depends upon obtaining the correct length of peptides and correct folding to mimic native structures (Shapira, *et al.*, 1984).

1.2.3 Viral Chemotherapy

Even with promising advances in the area of vaccines in recent years, it is apparent that active stimulation of the immune response does not solve all of the problems encountered in the control of viral infection and can even complicate therapy.

The risk of complications caused by administration of vaccines is in general so greatly outweighed by its preventative potential that vaccines are considered totally acceptable. This does not, however, prevent some hesitation when there is an awareness of possible complications after such immunisations as vaccinia. There is therefore a general reluctance for immunisation against diseases considered by many to be of remote threat, particularly the use of live vaccines, when there may be fear of reversion to a pathogenic form or incorporation of the genome into host DNA.

When an effective and undeniably safe vaccine is developed, trials are often very time-consuming when compared to other drug trials, a disadvantage when new viral diseases such as AIDS appear. A previously developed broad-spectrum antiviral compound could well prove to be active in such circumstances - enabling the disease to be tackled immediately, long before vaccine development can get under way.

The purely preventative nature of vaccination also gives us little hope of controlling viral infection once it has been contracted. This, together with the

now widespread use of cancer chemotherapy and organ transplantation resulting in a higher incidence of immunocompromised patients, means that ideally a range of antiviral chemotherapeutic agents will be available for use in combination with prophylactic measures, or when such measures cannot be relied upon.

1.3 ANTIVIRAL AGENTS AND THEIR TARGETS

1.3.1 Viral Adsorption and Cell Penetration

A recent approach to the inhibition of viral penetration into host cells has utilised suppression of the activity of a viral protein rather than a viral enzyme (Choppin, *et al.*, 1984). Myxo- and paramyxoviruses have been inhibited by oligopeptides with similar sequences to an essential polypeptide (F or fusion protein) involved in viral penetration. The protein involved has membrane-fusing activity upon activation by a host cell protease to give the F protein a new N-terminus which is highly conserved in the virus. The synthetic oligopeptides with similar hydrophobic sequences inhibit the F protein activity, the most active being carbobenzoxy-D-Phe-L-Phe-Gly-D-Ala-D-Val-D-Ile-Gly (50% inhibition at 0.02 μM against measles virus) and carbobenzoxy-D-Phe-L-Phe-Gly (50% inhibition at 0.2 μM). Research in this area will at least enable a better understanding of viral

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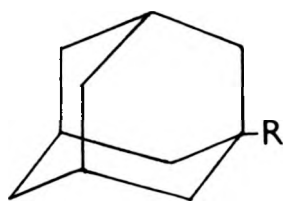
interaction with host cellular membrane receptors and may lead to compounds active *in vivo*.

1.3.2 Uncoating

1.3.2.1 Adamantane Derivatives



I

Fig. 1.3.2.1 Structure of adamantane derivatives.

1-Amino adamantane (amantadine, symmetrel) (I, R = NH_2) has been rather under-used clinically, despite being the only therapeutic or prophylactic treatment for influenza A infections in recent years. Amantadine acts, without being metabolised, at some point within the process of virus uncoating after the virus has entered the cell, although the exact point of attack remains to be unequivocally assigned (Skehel, *et al.* 1978; Oxford and Galbraith, 1980). The point of action may be on the pH control of cells, an acidic pH being required for fusion of the viral and vacuole membranes after viropexis (see Section 1.1.3).

1-Adamantane-methylamine (rimantadine; I, R = $CH(CH_3)NH_2$) has a similar mechanism of action, is used clinically in the USSR, and has been reported as being more effective (Burlington, *et al.*, 1974) with less side-effects on the central nervous system (Oxford, 1984). Tromantadine (I, R = $NHCOCH_2O(CH_2)_2NMe_2$) has some activity against HSV-1 and HSV-2.

The occurrence of amantadine and rimantadine resistant mutants has prompted studies with simultaneous administration of inactivated HN vaccine and amantadine (Webster, *et al.*, 1985). Such combinations could be valuable against highly pathogenic influenza outbreaks.

1.3.2.2 2'-Deoxyglucose

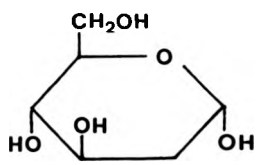


Fig. 1.3.2.2 2'-Deoxyglucose

HSV-1 infectivity is decreased by 2'-deoxy-glucose as a result of a reduced ability to penetrate the cell or in the uncoating process (Spivak, *et al.*, 1982), cellular attachment is little-affected. In

the case of influenza virus the target is known to be glycosylation of virus proteins in infected cells which is inhibited (Blough and Guitolini, 1979) altering their functioning and interaction with the cell membrane prior to budding and virus release.

1.3.2.3 Arildone and Derivatives

Activity against DNA and RNA viruses is exhibited by the β-diketone Arildone (1) (Diana, *et al.*, 1977). The mode of action of this class of compounds has been elucidated for polio and HSV-2, acting at an early stage, likely to be the uncoating of the virus, preventing production of virally coded RNA, DNA or proteins (McSharry, 1979). Arildone has shown promise against human cytomegalovirus replication, blocking at a stage after virion uncoating but prior to DNA synthesis (Tyms, *et al.*, 1984).

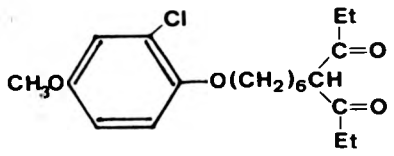
Also reported are the pyrazole analogues (2) with antiherpes activity (Diana, *et al.*, 1981) and the phosphonate derivative (3) (Diana, *et al.*, 1984) which was active *in vitro* and *in vivo* against HSV-2.

1.3.3 Intracellular Events

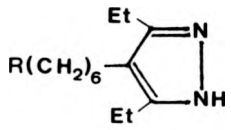
1.3.3.1 Synthetic Oligonucleotides

Current knowledge of the intracellular events following virus infection is rapidly increasing and this will surely result in new targets for drug development.

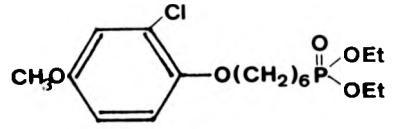
Specific interference with viral genomes to



(1)



(2)



(3)

Fig. 1.3.2.3 Arildone and analogues.

affect replication and/or transcription is a possibility. Sequence-specific non-polar oligonucleotides (Ts'O, *et al.*, 1983) may overcome problems associated with reaching the desired site such as penetration and enzymatic degradation. A decrease in polarity may be achieved using phosphotriesters between nucleosides rather than diester linkages.

1.3.3.2 The Interferons

After its discovery in 1957 by Isaacs and Lindenmann, the antiviral protein interferon was thought to show great promise as a clinically useful antiviral agent. However, difficulty in the production of sufficient quantities for study resulted in significant loss in interest until recent years. The

timely development of recombinant DNA technology and monoclonal antibodies has solved these initial problems and the different interferon (IFN) types have now been cloned and purified .

The three categories comprise IFN-α (leucocyte interferon), IFN-β (fibroblast interferon) and IFN-γ (immune interferon). Binding to specific cell surface receptors induces a cascade of cellular events with the induction of a number of new enzymes; $(2'-5')(A)_n$ synthetase, and a 73 K protein kinase which require double-stranded RNA for their activation (Kerr and Brown, 1978; Marcus, 1982), and this has therefore been assumed to be an intermediate or a by-product in all DNA and RNA virus replicative cycles. The activated 2-5A synthetase catalyses the synthesis of short-lived oligonucleotides $(2'-5')pppA(pA)_n$ or (2-5)A, which in turn activate an endonuclease (RNase L) to destroy mRNA and thereby inhibit protein synthesis in virus-infected cells treated with interferon (Baglioni, 1983). Several (2-5)A analogues have been synthesised and their antiviral activities investigated (Torrence, *et al.*, 1984; Lesiak, *et al.*, 1983; Goswami, *et al.*, 1982). Compounds of this type are, however, relatively impermeable to cell membranes by virtue of their highly charged nature, and are also susceptible to hydrolysis by phosphodiesterases and dinucleoside polyphosphatases.

The 73 K protein kinase simultaneously inactivates the chain initiation factor eIF-2 (Lengyel,

1982) by phosphorylation. Other biochemical events include inhibition of methylation of the 5'-terminal guanosine cap of viral mRNA (Baglioni, 1979).

Interferons have the potential to be antiviral antibiotics with a broad spectrum of specific activity, furthermore no problems of resistance have been reported. Human IFN-α and -β have shown promise when used in high dosage early in infection. Interferon's multifaceted nature, however, means that its other properties may result in deleterious effects. It has antiproliferative properties and immune enhancing effects and is thought to have a role in some autoimmune diseases, multiple sclerosis, and more recently, AIDS.

1.3.3.3 Methylation of mRNA as a Target

Many viral and cellular messenger RNAs contain N-7 methylated 5'-terminal cap structures. The methyl group is derived from S-adenosylmethionine, to produce S-adenosylhomocysteine (SAH) as a by-product, which is cleaved by S-adenosylhomocysteine hydrolase. This enzyme is inhibited by 9-β-D-arabinosyladenine (ara-A), (S)-9-(2,3-dihydroxypropyl)adenine (Holý, 1982) and 3-deazaadenosine and its carbocyclic derivative (De Clercq and Montgomery, 1983). The SAH which accumulates is a potent inhibitor of S-adenosylmethionine-dependent methylation.

Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) has a similar structure to guanosine and inosine. It shows broad spectrum activity against

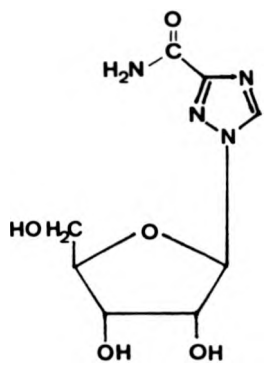


Fig. 1.3.3.3(i) Ribavirin

DNA and RNA viruses (Smith and Kirkpatrick, 1980). Ribavirin monophosphate inhibits IMP dehydrogenase (Streeter, *et al.*, 1973) and therefore affects GTP and dGTP pools. However, the fact that the related thiazole analogue inhibits IMP dehydrogenase but has minimal antiviral activity seems to rule out this route of specific antiviral action (Robins, *et al.*, 1982). The triphosphate, however, inhibits mRNA guanyltransferase (Goswami, *et al.*, 1979). The specific inhibition of influenza RNA polymerase (Eriksson, *et al.*, 1977) may well be due to inhibition of the essential 5'-cap transfer reaction required during influenza mRNA synthesis. Ribavirin analogues modified in the azole ring have met with some success, a highly potent and selective antiviral agent was

obtained on introducing selenium (Robins, *et al.*, 1983) (Fig. 1.3.3.3(ii)).

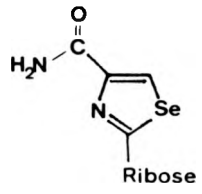


Fig. 1.3.3.3(ii)

The precise mechanism of action of ribavirin and its analogues is therefore still unclear, its broad spectrum activity seems most likely to be due to a number of different points of attack. Inhibition of protein synthesis cannot be ruled out, perhaps occurring by virtue of the similarity to 3-amino-1,2,4-triazole - a mitochondrial ribosome protein synthesis inhibitor.

1.3.3.4 Protein Synthesis and Processing as a Target

Apart from the interferons and their inducers, there are some other compounds having an effect on protein synthesis. Isatin-β-thiosemicarbazide, for example, inhibits late protein synthesis in poxvirus infected cells (Cooper, *et al.*, 1979).

Evidence that virus-infected cell membranes

have altered permeabilities and allow transport of certain compounds not normally taken up (Carrasco and Smith, 1980) led to investigation of the GTP methylene analogue $GppCH_2p$, which selectively inhibits protein synthesis in virus-infected cells only (Carrasco, 1978). This "membrane leakiness" is exhibited by a number of viruses including herpesviruses and paramyxoviruses, however, with the exception of vaccinia virus (Carrasco and Esteben, 1982), this is usually late in infection which may prove to be a drawback when the potential for antiviral exploitation of these membrane permeability changes is examined.

The interdependent manner of the sequential formation of HSV α, β and γ proteins (Honess and Roizman, 1974) may be exploitable. Gamma-polypeptides are able to shut off the synthesis of β-proteins (including thymidine kinase and DNA polymerase). The development of small, stable polypeptides able to interact with the γ-protein receptors could lead to prevention of the synthesis of these viral-specific enzymes.

1.3.4 Viral Enzyme Inhibition

Perhaps the best and most widely investigated targets for antiviral chemotherapy are the viral-specific enzymes. Recent progress has been in the identification and characterisation of a number of virus-coded enzymes which are either absent from uninfected cells or

Virus	Ribonucleotide Reductase	Protein Kinase	Thymidine Kinase	DNA Pol	RNA Pol	DNase	RNase	N
HSV-1	+	+	+	+	-	+	-	-
HSV-2	+	?	+	+	-	+	-	-
VZV	?	?	+	+	-	+	-	-
EBV	+	?	-	+	-	+	-	-
CMV	?	?	-	+	-	+	-	-
Influenza	-	+	-	-	+	-	+	+
Rhino	-	-	-	-	+	-	+	-

Table 1.3.4 Known viral enzymes.

(from Öberg & Johansson J. Antimicrob. Chemother. **14** A, 6 1984)

fundamentally different from their normal cellular counterparts.

Enzymes with a unique activity include the RNA transcriptase of influenza and parainfluenza, and the reverse transcriptase of RNA oncogenic viruses. Negative sense RNA viruses such as influenza have associated with the virion a RNA dependent RNA polymerase (transcriptase) required for the synthesis of early viral mRNAs.

Virally encoded enzymes are also known which catalyse reactions occurring in uninfected cells but which have sufficiently different properties to the corresponding cellular enzymes to allow selective inhibition by chemotherapeutic agents.

Viruses of the herpes group are known to code for at least six such enzymes which are required for their own biosynthetic pathways; ribonucleotide reductase, thymidine kinase, DNase, uracil-DNA-glycosylase, dUTPase and DNA polymerase (see Table 1.3.4).

1.3.4.1 Thymidine Kinase

Herpesvirus encoded thymidine kinase is not yet a direct target for antiviral agents but is critical for the activation of a number of nucleoside analogue antiviral compounds which are not substrates for the cellular enzyme.

The virus induced thymidine kinase differs from the host cell enzyme in molecular weight, electrophoretic mobility, isoelectric point and

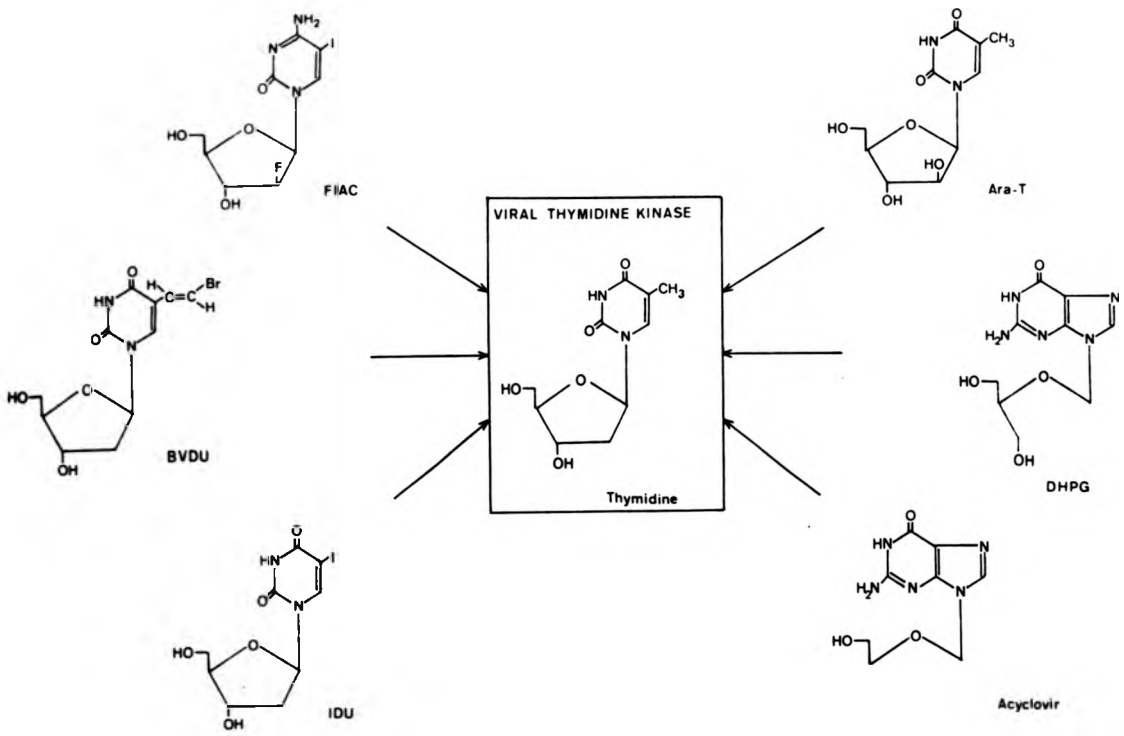


Fig. 1.3.4.1 Nucleoside analogue substrates for herpesvirus thymidine kinase.

immunological properties (Kit, 1979). The viral enzyme has rather poor substrate specificity and is therefore a prime target for the selective activation of antiviral compounds. The HSV-1 thymidine kinase, for example, phosphorylates a number of nucleoside analogues as well as thymidine (Fig. 1.3.4.1).

1.3.4.2 Acyclonucleoside Analogues

Interest in a class of antiviral nucleoside analogues in which major modification of the sugar residue is present was initiated by the discovery of 9-(2-hydroxyethoxymethyl)guanine (acyclovir) as a potent agent against HSV-1 and HSV-2 *in vitro* (Schaeffer, *et al.*, 1978), active at concentrations as low as 0.1 μM with no cytotoxicity up to 20 mM. Acyclovir has rapidly progressed to clinical use and is now available for treatment of HSV-1 occular keratitis, genital herpes and varicella zoster lesions.

The drug has been shown to be phosphorylated to its monophosphate by the herpesvirus thymidine kinase, the monophosphate is then further phosphorylated by cellular kinases to the triphosphate (Miller and Miller, 1982) which preferentially inhibits the viral DNA polymerase, and can be incorporated into oligonucleotides resulting in chain termination by virtue of the availability of only one phosphodiester forming hydroxyl group (Furman, *et al.*, 1979, 1980).

Some close analogues of acyclovir have been synthesised and shown to have increased potency and

similar mechanisms of action. Of these compounds, the most promising have been 9-(1,3-dihydroxy-2-propoxymethyl)guanine (2'NDG, DHPG, Fig. 1.3.4.1) (Smith, *et al.*, 1981) and (S)-9(2,3-dihydroxy-1-propoxymethyl)-guanine (iNDG) (MacCoss, *et al.*, 1985). More recently a cyclic GMP analogue, 9-[(2-hydroxy-1,3-2-dioxaphosphorinan-5-yl)oxymethyl]guanine P-oxide, sodium salt (2'-nor-cGMP, Fig. 1.3.4.2) has been reported as a potent and broad spectrum anti-DNA-viral agent (Tolman, *et al.*, 1985). Unlike other acyclonucleosides 2'-nor-cGMP activity against herpesviruses is not dependent on virus-induced thymidine kinase activity, although cleavage of the cyclic phosphate intracellularly could produce 2'NDG monophosphate, which could be converted to the triphosphate by host enzymes. Intracellular levels of 2'NDG triphosphate are, however, insufficient to account for activities observed, indicating a mechanism separate from that of the known acyclonucleosides.

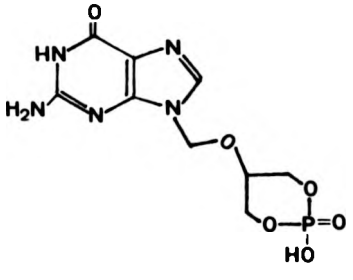


Fig. 1.3.4.2 2'-Nor-cGMP

1.3.4.3 5-Iodo-2'-deoxyuridine (Idoxuridine, IDU)

IDU (Fig. 1.3.4.1) is phosphorylated by

both virus-infected and normal cells and subsequently incorporated into DNA (Prusoff and Goz, 1973) whereby replication and transcription are affected. Analogues are reported as showing selectivity against herpesviruses by virtue of selective phosphorylation by the viral thymidine kinase (DeClercq, *et al.*, 1978). Since IDU and its close analogue trifluorothymidine (TFT) show little specificity for incorporation into DNA they can only be used where cellular DNA synthesis is minimal, as the presence of nucleoside analogues in DNA has an effect on transcription and translation (Otto, *et al.*, 1982).

1.3.4.4 E-5-(2-Bromovinyl)-2'-deoxyuridine (BVDU)

BVDU (Fig. 1.3.4.1) is the most effective antiviral agent of a number of 5-(2-halogenovinyl)-2'-deoxyuridine derivatives (DeClercq, *et al.*, 1979, 1980). BVDU is a potent inhibitor of HSV-1 but is less effective against HSV-2. The mode of action has been suggested to be via specific phosphorylation to the 5'-phosphate in HSV-1 and HSV-2 infected cells by the viral thymidine kinase (Cheng, *et al.*, 1981), followed by further phosphorylation to the 5'-pyrophosphate by the dTMP kinase activity of HSV-1 thymidine kinase but not by the HSV-2 enzyme. Subsequent phosphorylation by cellular enzymes to the 5'-triphosphate then occurs within HSV-1 infected cells. In HSV-2 infected cells, however, the viral kinase phosphorylates the nucleoside only to the 5'-monophosphate (Descamps and

DeClercq, 1981), which can then be attacked by cellular thymidylate kinase, for which BVDU-monophosphate is a good substrate, before further phosphorylation by a cellular kinase can occur (Barr, *et al.*, 1983).

The BVDU 5'-triphosphate shows marked preferential inhibition of HSV DNA polymerase (Allaudeen, *et al.*, 1981), and is also incorporated into viral DNA resulting in an increased lability of the DNA as revealed by an increased number of single strand breaks observed in alkaline sucrose gradients (Mancini, *et al.*, 1983). The extent of incorporation is related to antiviral activity and occurs at much lower concentrations than required by cellular DNA. BVDU also appears to interfere with the glycosylation of HSV-1 late polypeptides (Siegel, *et al.*, 1984).

The drawbacks with this highly potent compound arise from the unusual base structure, which, should it become detached from the sugar, perhaps by the action of a nucleoside phosphorylase, is likely to have unforeseen side-effects on host cellular DNA or RNA synthesis.

BVDU and similar compounds are also likely to be phosphorylated to some extent in uninfected cells and therefore incorporated into cellular DNA. The presence of the abnormal halogenated base is likely to affect the base pairing properties of a growing DNA chain with possible mutagenic consequences. Also likely to be affected, is the behaviour of enzymes

in which base recognition is important.

1.3.4.5 2'-Fluoro-5-iodo-1-β-D-arabinofuranosyl-cytosine (FIAC)

FIAC (Fig. 1.3.4.1) also relies upon specific phosphorylation for its antiviral activity, the 5'-triphosphate is able to interfere with the viral DNA polymerase or be incorporated into DNA, probably resulting in defective mRNA and therefore proteins. Inhibition of viral DNA polymerase is also likely to be of importance (Larsson and Öberg, 1981).

1.3.4.6 Ribonucleotide Reductase

Ribonucleotide reductase is an essential enzyme for DNA synthesis in all prokaryotic and eukaryotic cells, catalysing the synthesis of all four deoxyribonucleotides by direct reduction of the corresponding ribonucleotides.

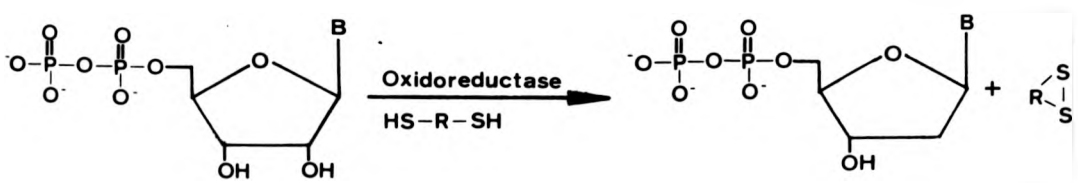


Fig. 1.3.4.6 Action of ribonucleotide reductases.

A number of herpesviruses induce a ribonucleotide reductase activity which differs from that of the host cell. These include HSV-1 (Cohen, 1972), HSV-2 (Cohen, *et al.*, 1974), pseudorabies virus (PrV) (Lankinen, *et al.*, 1982) and EBV (Henry, *et al.*, 1978). The HSV-1 enzyme is known to be virus-coded (Dutia, 1983) and like all other enzymes catalyses the reduction of all ribonucleotides.

Inhibition of ribonucleotide reductase by α-(N)-heterocyclic carboxaldehyde thiosemicarbazones has been reported (Moore and Sartorelli, 1984), these compounds being very powerful inhibitors of the mammalian enzyme, a property leading to potential anti-viral activity. A good correlation is indeed observed between the inhibition of the formation of herpesvirus virions and the inhibition of the virus induced reductase by compounds of this type (Brockman, *et al.*, 1970).

Active compounds are heteroaromatic rings linked to a thiosemicarbazide side chain through the carbon atom α- to the ring nitrogen (Fig. 1.3.4.6(i)). High selectivity against HSV-1 and HSV-2 has been

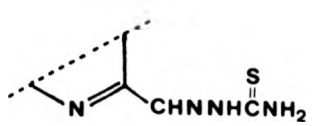
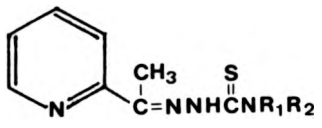


Fig. 1.3.4.6(i)

observed for a series of 2-acetylpyridine thiosemicarbazones, with the unusual property of higher activity against HSV-2 (Shipman, *et al.*, 1981) (Fig. 1.3.4.6(ii).



R_1	R_2
H	H
H	1-Adamantyl
CH_3	CH_3

Fig. 1.3.4.6(ii)

Inhibition of vaccinia and some parainfluenza viruses has been observed for analogues of methisazone (N-methylisatin—β-thiosemicarbazone (Fig. 1.3.4.6(iii) (Giovanninetti, *et al.*, 1981), the mechanism of action is unclear however.

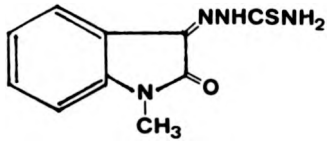


Fig. 1.3.4.6(iii) Methisazone

1.3.4.7 Protein Kinase

An envelope-associated cyclic AMP-independent protein kinase has been reported for HSV-1 (Lemaster and Roizman, 1980; Blue and Stubbs, 1981). It is unclear, however, whether the enzyme is virally

coded, host cell derived or a virus-induced host cell enzyme. The protein kinase could be a valuable target for chemotherapy if its role in the sequential synthesis of α, β and γ HSV-polypeptides is confirmed.

1.3.5 Viral Nucleic Acid Polymerase Inhibitors

Almost all viruses have a specific nucleic acid polymerase activity (Table 1.3.5), many of which are easily assayed in cell-free systems and present excellent primary targets for antiviral chemotherapy.

Several of the nucleoside analogues already discussed rely upon selective activation by a herpesvirus thymidine (or deoxycytidine) kinase activity before they may act upon the DNA polymerase and other targets. The antiviral compound vidarabine (9-β-D-arabinofuranosyl(adenine) ara-A) is phosphorylated by cellular kinases to the 5'-triphosphate which is a preferential inhibitor of HSV DNA polymerase (Drach and Shipman, 1977). Ara-A also inhibits ribonucleotide reductase, RNA-dependent RNA polymerase and some other enzyme systems.

It should also be noted that cytomegalovirus does not induce a thymidine kinase activity (Zavada, *et al.*, 1976), and outside the herpesvirus group only vaccinia has been shown to code for the enzyme. These compounds are therefore not broad spectrum antiviral agents and require metabolism before they become active. There is therefore a need for compounds able to act

Virus	Enzyme activity
Picornaviruses	
Poliovirus, EMC, FMDV, rhinovirus	RNA-dependent RNA polymerase, EC 2.7.7
Poliovirus, FMDV	endonuclease, EC 3.1.4
Poliovirus, EMC, FMDV	peptide hydrolase (protease), EC 3.4
Reoviruses	
Reovirus	(guanine-7)-methyltransferase, EC 2.1.1
Reovirus, rotavirus	RNA-dependent RNA polymerase, EC 2.7.7
Reovirus	poly G polymerase, EC 2.7.7
Reovirus	poly A polymerase, EC 2.7.7
Reovirus	mRNA guanylyltransferase, EC 2.7.7
Reovirus	nucleoside triphosphatase, EC 3.6.1
Reovirus	polynucleotide 5'-triphosphatase, EC 3.6.1
Reovirus	triphosphate-pyrophosphate exchange
Togaviruses	
Sindbis virus, Semliki Forest virus	protein kinase, EC 2.7.1
Sindbis virus, Semliki Forest virus	RNA-dependent RNA polymerase, EC 2.7.7
Sindbis virus	endonuclease, EC 3.1.4
Ortomyxoviruses	
Influenza A virus	protein kinase, EC 2.7.1
Influenza A virus	nucleoside diphosphate kinase, EC 2.7.4
Influenza A, B, C virus	RNA-dependent RNA polymerase, EC 2.7.7
Influenza A, B, virus	neuraminidase, EC 3.2.1
Influenza A virus	nucleoside triphosphatase, EC 3.6.1
Paramyxoviruses	
NDV	(guanine-7)-methyltransferase, EC 2.1.1
Sendai virus	protein kinase, EC 2.7.1
NDV, mumps virus, measles virus, Sendai virus, SV5	RNA-dependent RNA polymerase, EC 2.7.7
NDV, Sendai virus, SV5	poly A polymerase, EC 2.7.7
NDV	mRNA guanylyltransferase, EC 2.7.7
Sendai virus	endonuclease, EC 3.1.4
NDV, mumps virus, Sendai virus	neuraminidase, EC 3.2.1
NDV	adenosine triphosphatase (ATPase), EC 3.6.1
Rhabdoviruses	
VSV	(guanine-7)-methyltransferase, EC 2.1.1
VSV	(adenine-2)-methyltransferase, EC 2.1.1
VSV, rabies virus	protein kinase, EC 2.7.1
VSV	nucleoside diphosphate kinase, EC 2.7.1
VSV, rabies virus, salmonid viruses	RNA-dependent RNA polymerase, EC 2.7.7
VSV	mRNA guanylyltransferase, EC 2.7.7
VSV	poly A polymerase, EC 2.7.7
VSV	endonuclease, EC 3.1.4
VSV	nucleoside triphosphatase, EC 3.6.1
Retroviruses	
ASV, MuLV, MTV, RSV, FeLV, AMV	protein kinase, EC 2.7.1
RSV, AMV, etc.	RNA-dependent DNA polymerase, (reverse transcriptase), EC 2.7.7
RSV, AMV, etc.	DNA-dependent DNA polymerase, (reverse transcriptase), EC 2.7.7
AMV, RSV	endonuclease, EC 3.1.4
RLV	RNase H, EC 3.1.4
RLV	protease, EC 3.4
AMV	nucleoside triphosphatase, EC 3.6.1
Arenaviruses	
Pichinde virus	RNA-dependent RNA polymerase, EC 2.7.7
Bunyaviruses	
Uukuniemi virus	RNA-dependent RNA polymerase, EC 2.7.7

Avian myeloblastosis virus, AMV; avian sarcoma virus, ASV; encephalomyocarditis virus, EMC; feline leukemia virus, FeLV; foot and mouth disease virus, FMDV; mammary tumor virus, MTV; murine leukemia virus, MuLV; Newcastle disease virus, NDV; Raucher leukemia virus, RLV; Rous sarcoma virus, RSV; simian virus 5, SV5; vesicular stomatitis virus, VSV.

Table 1.3.5 Enzymes induced by RNA viruses.

Helgstrand & Öberg , 1980

Virus	Enzyme activity
Parvoviruses	
Kilham rat virus	DNA-dependent DNA polymerase, EC 2.7.7
Papovaviruses	
Polyoma virus	protease, EC 3.4
Adenoviruses	
Adenovirus type 2	protein kinase, EC 2.7.1
Adenovirus type 2	protease, EC 3.4
Herpesviruses	
HSV-1, HSV-2	ribonucleotide reductase, EC 1.17.4
HSV-1, pseudorabies virus	protein kinase, EC 2.7.1
HSV-1, HSV-2, VZV, EBV, pseudorabies virus, HVT, EHV-1	nucleoside phosphotransferase (thymidine kinase), EC 2.7.1
HSV-1	thymidylate kinase, EC 2.7.4
HSV-1, HSV-2, VZV, HVT, MDV, CMV, EBV, pseudorabies virus, EHV-1	DNA-dependent DNA polymerase, EC 2.7.7
HSV-1, HSV-2, VZV, CMV, EBV	DNase, EC 3.1.4
HSV-1	Deoxycytidine deaminase, EC 3.5.4
Iridoviruses	
Frog virus 3	protein kinase, EC 2.7.1
ASFV	DNA-dependent DNA polymerase, EC 2.7.7
Frog virus 3	DNase, EC 3.1.4
Frog virus 3	RNase, EC 3.1.4
Frog virus 3	adenosine triphosphatase (ATPase), EC 3.6.1
Poxviruses	
Vaccinia virus	(guanin-7)-methyltransferase, EC 2.1.1
Vaccinia virus	mRNA ribose-2′-*O*-methyltransferase, EC 2.1.1
Vaccinia virus	protein kinase, EC 2.7.1
Vaccinia virus	nucleoside phosphotransferase (thymidine kinase), EC 2.7.1
Vaccinia virus, rabbit pox virus	DNA-dependent RNA polymerase, EC 2.7.7
Vaccinia virus	DNA-dependent DNA polymerase, EC 2.7.7
Vaccinia virus	poly A polymerase, EC 2.7.7
Vaccinia virus	mRNA guanylyltransferase, EC 2.7.7
Vaccinia virus	DNase, EC 3.1.4
Vaccinia virus	nucleoside triphosphatase, EC 3.6.1
Vaccinia virus	polynucleotide 5′-triphosphatase, EC 3.6.1
Vaccinia virus	polynucleotide ligase, EC 6.5.1
Hepatitis B viruses	
Human hepatitis B virus, woodchuck hepatitis B virus	DNA-dependent DNA polymerase, EC 2.7.7

African swine fever virus, ASFV; cytomegalo virus, CMV; Epstein-Barr virus, EBV; equine herpesvirus type 1, EHV-1; herpes simplex virus type 1, HSV-1; herpes simplex virus type 2, HSV-2; herpes-virus of turkeys, HVT; Marek's disease virus, MDV; varicella-zoster virus, VZV.

Table 1.3.5 Enzymes induced by DNA viruses

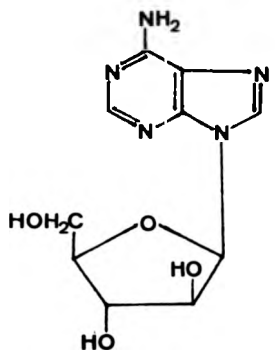


Fig. 1.3.5(i) Ara-A

directly on nucleic acid biosynthesis without being metabolised.

Nucleic acid biosynthesis can be regarded as essentially a four step process, the elements of which are outlined in Fig. 1.3.5(ii).

(i) Template binding; the polymerase enzyme recognises a specific template nucleotide sequence and binds to it.

(ii) Initiation; a nucleoside 5'-triphosphate or oligonucleotide primer binds to the enzyme/template complex at a site corresponding to the 5'-terminus of the nucleic acid to be synthesised.

(iii) Elongation; nucleoside 5'-triphosphates bind to the active site as determined by the template sequence, and react with the free 3'-hydroxyl group

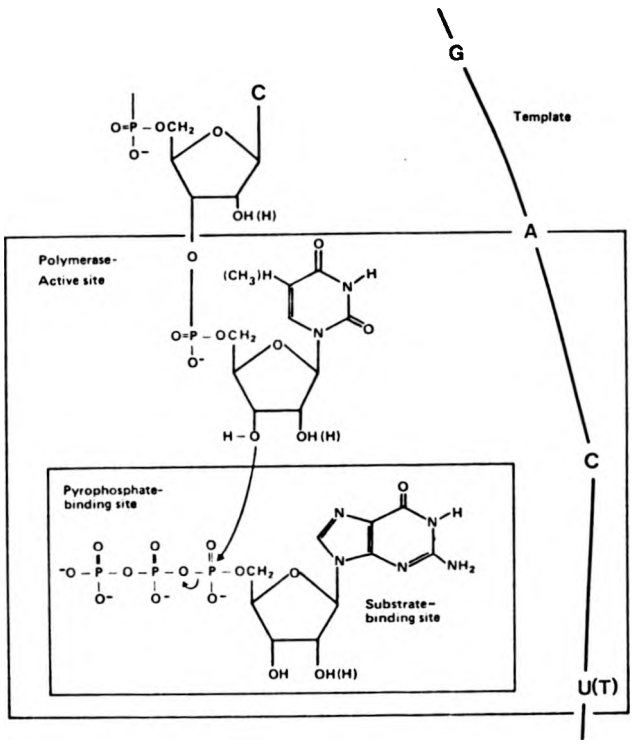


Fig. 1.3.5(ii) Stages in nucleic acid synthesis.

of the growing nucleic acid molecule, with the elimination of pyrophosphate via a pyrophosphate binding site.

(iv) Termination; nucleic acid synthesis ceases on reaching a specific template sequence and the complex of template, polymerase and nascent nucleic acid dissociates.

All four of the processes present a possible target for interference by chemotherapeutic agents. Since the agent must be selective, however, there must be differences in the viral and cellular enzyme active sites due to different amino acid sequences at essential positions. The principal targets for the design of antiviral molecules can be regarded not only as the substrates (nucleoside analogues) and templates or primers, but also the by-products of the reaction. Analogues of pyrophosphate are almost unique in that they act preferentially against viral polymerases without being metabolised in the cell, and in general show a broader spectrum of activity than those compounds discussed so far.

1.3.6 Pyrophosphate Analogues

It has now been known for some time that analogues of inorganic pyrophosphate can exhibit antiviral activity. The first compound of this type to be evaluated as a chemotherapeutic agent was phosphonoacetic acid (PAA, Fig. 1.3.6).

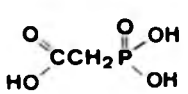


Fig. 1.3.6 Phosphonoacetic acid (PAA) (1).

PAA was discovered through random screening of compounds in herpesvirus-infected WI-38 tissue culture cells (Shipkowitz, *et al.*, 1973). Its chemotherapeutic potential was at first unexciting, with high concentrations (100 µg/ml) required for consistent activity in cell culture, although there were no adverse effects on cell growth or division at higher levels, and early studies appeared to show that only herpes simplex virus was susceptible.

PAA was subsequently shown to suppress replication of cytomegalovirus (Huang, 1975), Epstein-Barr virus (Yajima, *et al.*, 1976), varicella-zoster virus (Overby, *et al.*, 1977) and vaccinia virus in tissue culture. Promising results against ocular, cutaneous and encephalitic herpesvirus infections in animals have been reported (Gerstein, *et al.*, 1975; Meyer, *et al.*, 1976; Shipkowitz, *et al.*, 1973).

These *in vivo* studies led to further investigations on the molecular events in normal and HSV-infected human cells treated with PAA. The rates of normal cellular RNA, DNA and protein synthesis were not significantly affected by PAA (Overby, *et al.*, 1974), whereas in HSV-infected cells synthesis of virus-specific DNA was completely inhibited. Mao, *et al.*, (1975) isolated the virus-induced DNA polymerase from infected cells and showed direct inhibition by PAA on the partially purified HSV-induced enzyme.

	Polymerase					
Property	HSV-1	HSV-2	HCMV	α	β	γ
Function	Virus DNA Replication	Virus DNA Replication	Virus DNA Replication	Cellular DNA Replication	DNA Repair	Mitochondrial DNA Synthesis
M. Wt.	1.5-2.0 x 10^5	1.5 x 10^5	~1.5 x 10^5	1.1-1.8 x 10^5	3.5 x 10^4	1.1-3.3 x 10^5
pH optimum	8.0	8.0	8.0	7.3	7.9	8.5
Mg^{2+} concentration (mM)	3	3	10	5-10	5-10	5-10
Salt optimum	150 mM K_2SO_4	100 mM $(NH_4)_2SO_4$	120 mM KCl	20 mM KCl	0	100 mM KCl
[PAA] (μM) Producing 50% inhibition	0.4-7	1-2	0.4	35	> 100	> 100

Table 1.3.6.1 A comparison fo the functional roles and properties of mammalian cellular DNA polymerase and herpesvirus-induced enzymes.

1.3.6.1 DNA Polymerases

In clarifying the antiviral role of PAA it is helpful to compare the roles and properties of normal mammalian cellular DNA polymerases and the herpesvirus-induced enzyme. The cellular polymerases are necessary for cell division and DNA repair, while the virus-induced enzyme is required for vDNA replication.

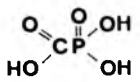
Of the three biophysically and biochemically distinct cellular DNA polymerases, α, β and γ (see Table 1.3.6.1), the herpesvirus induced enzyme is most similar to DNA polymerase α. Both enzymes are able to copy efficiently, double stranded DNase "activated" DNA as well as the synthetic primer-template $(dG)_{\approx 15}$-$(dC)_n$ (Wagner, *et al.*, 1974; Weissbach, *et al.*, 1973). The enzymes can only be separated by phosphocellulose chromatography (Weissbach, *et al.*, 1973). The virally induced enzymes are most active at high ionic strengths, at which host cell polymerases are totally inactive (Weissbach, *et al.*, 1973). DNA polymerase α is inhibited by PAA but at concentrations up to 100 times higher than required to inhibit the herpesvirus-induced enzymes (Bolden, *et al.*, 1975; Miller and Rapp, 1976; Sabourin, *et al.*, 1978), whereas DNA polymerase β and γ are relatively unaffected by high concentrations of PAA.

PAA resistant mutants of herpesvirus strains have been isolated and characterised (Honess and Watson, 1977; Powell and Purifoy, 1977) and have shown

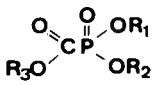
unequivocally that PAA inhibits herpesvirus replication by direct interaction with and inactivation of the viral DNA polymerase.

1.3.6.2 Mechanism of Action and Structure-Activity Relationships

Independent studies by Helgstrand, *et al.* (1978) and Reno, *et al.* (1978) led to the evaluation of phosphonoformic acid (PFA) (2) (Fig. 1.3.6.2) as an antiviral compound, which was found to inhibit herpesvirus DNA polymerase and herpesvirus replication in a manner similar to PAA.



(2)



(3)

Fig. 1.3.6.2 Phosphonoformic acid (2) and esters (3).

The activity of PFA revealed that separation of the phosphono and carboxyl group negative charges is not mandatory for antiherpes activity, and subsequently, despite the structure of PAA providing little opportunity for chemical modification to optimise activity, a number of analogues have been synthesised and tested on isolated viral enzymes in cell culture and animal models (Table 1.3.6.2).

Analogs of phosphonoacetate tested for antiherpesvirus activity

Analog	Activity	Reference
I. Carboxyl esters		
Ethyl; propyl; *t*-butyl	Yes (ethyl)	Herrin et al. (1977)
Cyclohexyl; octyl; benzyl	Yes (octyl)	Herrin et al. (1977)
II. Monophosphate esters		
Methyl; propyl; hexyl	No	Herrin et al. (1977)
III. Triesters		
Trimethyl	No	Lee et al. (1976)
Triethyl	No	Shipkowitz et al. (1973)
IV. Monophosphinic acids		
Phenyl; 4-methoxyphenyl; methyl	No	Herrin et al. (1977)
V. Phosphono analogs		
Sulfoacetate	No	Leinbach et al. (1976)
Malonate	No	Leinbach et al. (1976)
Arsenoacetate	Yes	Newton (1979)
VI. Carboxyl analogs		
Phosphonoacetaldehyde	No	Boezi (1979)
Phosphonoacetamide	No	Boezi (1979)
N-Methylphosphonoacetamide	No	Boezi (1979)
N-Propyl-; *N*-butyl-; *N*-cyclohexyl-; *N*-amantylphosphonoacetamide	Yes	von Esch (1978)
Acetonylphosphate	No	Boezi (1979)
Aminomethylphosphonate	No	Lee et al. (1976)
α-Amino ethyl phosphonate	No	Lee et al. (1976)
N-(phosphonoacetyl)-L-aspartate	No	Boezi (1979)
Methylene diphosphonate	No	Lee et al. (1976)
Methylene diarsonate	Yes	Newton (1979)
Arsonomethylphosphonate	Yes	Newton (1979)
VII. Methylene analogs		
Phosphonoformate	Yes	Reno et al. (1978)
Phosphonopropionate	No	Shipkowitz et al. (1973)
Phosphonobutyrate	No	Shipkowitz et al. (1973)
α-Phosphonopropionate	No	Leinbach et al. (1976)
α-Methyl-2-phosphonopropionate	No	Leinbach et al. (1976)
α-Phenylphosphonoacetate	No	Leinbach et al. (1976)
α-Aminophosphonoacetate	No	Leinbach et al. (1976)
VIII. Other related compounds		
Phosphoglycolate	No	Leinbach et al. (1976)
Imidodiphosphonate	No	Boezi (1979)
Carbamyl phosphate	No	Boezi (1979)
2'-Deoxyribothymidine-5'-phosphorophosphonoacetate	No	Boezi (1979)
Purine-5'-mono-carboxymethyl-phosphonate	Yes	Heimer and Nussbaum (1977)
Pyrimidine-5'-mono-carboxymethyl-phosphonate	Yes	Heimer and Nussbaum (1977)

Table 1.3.6.2 PAA analogues and their antiherpes activity. (From Overby, 1982)

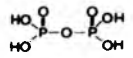
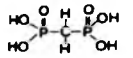
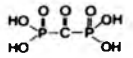
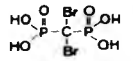
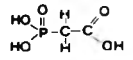
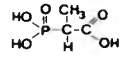
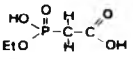
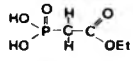
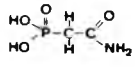
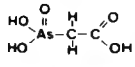
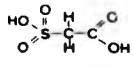
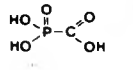
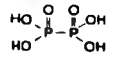
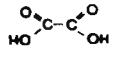
Compound	Concentration(μM) giving 50% inhibition				
	HSV-1 DNA-pol	HSV-2 DNA-pol	HCMV DNA-pol	Influenza RNA-pol	Cellular DNA-polα
	>500	>500	>500	450	>500
	>500	—	>500	>500	>500
	10	27	6	280	100
	>500	>500	—	10	350
	0·5	0·7	0·4	300	35
	15	550	18	>500	>500
	>500	>500	>500	>500	>500
	50	—	—	—	>500
	>500	>500	—	>500	>500
	—	270	—	—	—
	>500	—	—	—	>500
	0·3	0·5	0·3	30	40
	150	12	150	>500	250
	115	400	130	>500	>500

Table 1.3.6.3 Structures of some pyrophosphate analogues and their reported inhibitory properties.

Substitution of the phosphono group by carboxyl and sulphono groups results in loss of activity against the DNA polymerases induced by the herpesvirus of turkeys (Leinbach, *et al.*, 1976). Replacement of the phosphono and/or carboxyl group by arseno groups results in comparable activities for methylenebisarsonate and arsenoacetic acid in plaque reduction assays with HSV-1 (Newton, 1979) but no activity is observed *in vivo* (Mao, *et al.*, 1985). Substitution of the carboxyl group by moieties other than arseno- or phosphono-generally abolishes antiviral activity, with the exception of some amide derivatives (Mao, *et al.*, 1985). Replacement of the carboxyl group of PAA by a phosphono-group yields methylenebisphosphonate, which, like pyrophosphate, is devoid of significant antiherpes activity (Eriksson, *et al.*, 1980), carbonylbisphosphonate however, shows significant inhibition of HSV-1 DNA polymerase (Eriksson, *et al.*, 1980).

Substitution of the methylene group of PAA with various alkyl or aryl groups resulted in a reduction in activity against the polymerase enzyme and reduced inhibition of HSV-1 plaque formation. In general esters have proved to be inactive relative to PAA and PFA, including aliphatic and aromatic mono-, di- and triesters of PFA (Norén, *et al.*, 1983). None showed inhibition of HSV-1 DNA polymerase *in vitro*. Effective antiherpes activity was, however, observed

for several compounds, probably due to hydrolysis by non-specific esterases. More recently, Mao, *et al.* (1985) reported that small alkyl esters such as methyl, ethyl and propyl, only slightly decrease in activity against the HSV-induced DNA polymerase, suggesting that, contrary to earlier reports, an ionisable carboxylic acid is not an absolute requirement for activity. Compounds of this type, and bridge substituted PAA, would be expected to traverse the cell membrane with more ease than the unesterified compound, none were more active than the parent compounds however.

1.3.6.3 Mechanism of Action of PAA

PAA inhibits herpesvirus DNA polymerase by interacting with the proposed pyrophosphate binding site at the enzyme active site (Leinbach, *et al.*, 1976; Mao and Robishaw, 1975). Non-competitive inhibition is exhibited by both PAA and pyrophosphate against the virus-induced enzyme with respect to the four deoxyribonucleoside triphosphates and non-competitive inhibition with respect to DNA at low deoxyribonucleoside triphosphate concentrations but almost uncompetitive inhibition at high concentrations.

Inhibition studies with pyrophosphate gave apparent inhibition constants in the mM range, whereas for PAA values of 1-2 μM were obtained. PAA is a competitive inhibitor of pyrophosphate in the pyrophosphate-deoxyribonucleoside 5'-triphosphate exchange reaction

and in multiple inhibition analyses pyrophosphate and PAA function as mutually exclusive inhibitors and hence bind at the same site. Additional evidence for the shared binding site of these compounds comes from the observation that PAA resistant mutants of HSV are also more resistant to PFA and pyrophosphate (Eriksson and Öberg, 1979; Hay and Subak-Sharpe, 1976; Reno, *et al.*, 1978).

An alternate product mechanism has been used to describe the inhibition patterns observed with PAA (Leinbach, *et al.*, 1976). Pyrophosphate is predicted to act as a substrate for the reverse reaction of the polymerisation, i.e., pyrophosphate-deoxyribonucleoside 5'-triphosphate exchange. However, nucleotides containing [2-^{3}H]PAA have not been recovered from DNA polymerisation reactions containing [2-^{3}H]PAA (Cload, 1983) and, furthermore, nucleotide analogues such as dAMP-PAA and dTMP-PAA are neither substrates nor inhibitors of HSV DNA polymerase (Cload, 1983; Boezi, 1979).

Further insight into the mechanism of action of pyrophosphate analogues has come from a consideration of their metal-binding abilities. DNA and RNA polymerases are zinc-requiring enzymes (Mildvan and Leob, 1979) as is reverse-transcriptase (Poesz, *et al.*, 1974) which is inhibited by PFA (Sundquist and Öberg, 1979). The stability constants for some metal complexes of PAA and other pyrophosphate analogues have been

determined (Stünzi and Perrin, 1979; Cload and Hutchinson, 1983) and both PAA and PFA found to form strong complexes with zinc in particular. It has been suggested that these compounds are active by virtue of their ability to chelate with an essential metal ion in the herpes DNA polymerase (Perrin and Stünzi, 1981).

As shown by the spectrum of activity of PAA and PFA (Table 1.3.6.3(i), as well as inhibiting herpesvirus DNA polymerase and the reverse transcriptases, PFA in particular is also an effective inhibitor of influenza virus RNA polymerase activity (Helgstrand, *et al.*, 1978; Stridh, *et al.*, 1979).

Inhibition of polymerases by PFA and PAA

Enzyme	Concentration giving 50 % inhibition, μM	
	PFA	PAA
RNA polymerases		
Influenza virus (Mg^{2+})	20	300
Influenza virus (Mn^{2+})	0.3	0.9
VSV	500	-
Reovirus	>500	-
Calf thymus I	>500	>500
Calf thymus II	>500	>500
E. coli	>500	>500
Reverse transcriptases		
AMV	7	4,000
RMuLV	0.7	600
DNA polymerases		
HSV-1 (strain C 42)	0.4	0.5
HSV-1 (strain 124)	3.5	7.0
Hepatitis B virus	20	>500
Calf thymus α (25 U/assay)	50	75
Calf thymus α (2.5 U/assay)	3.5	6.5
Calf liver γ	>500	>500
Micrococcus luteus	>500	>500
E. coli	>500	>500

Table 1.3.6.3(i) Inhibition of polymerases by PAA and PFA.

The RNA polymerase activity of influenza virus is known to be zinc requiring (Oxford and Perrin, 1977) and subsequent studies on the inhibition of influenza RNA transcriptase systems has provided the best evidence supporting the hypothesis that pyrophosphate analogues inhibit these enzyme activities by complexing with an essential zinc ion at the active site of the enzyme and thus inhibiting, either by preventing the binding of incoming nucleoside triphosphates or preventing the release of inorganic pyrophosphate once the internucleotide bond has been formed by the enzyme.

Recent studies on influenza virus have shown that PFA does not inhibit the initiation of mRNA formation, and in the presence of PFA the primer of about thirteen bases can be elongated with about twelve bases, but further elongation is inhibited (Stridh and Datema, 1984). It was suggested that after addition of the conserved nucleotides, changes in the viral core proteins may result in an increased sensitivity to pyrophosphate analogues by, for example, allowing zinc chelation, resulting in enzyme inhibition. Alternatively, a different protein such as PA may be involved in the synthesis of the message-specific sequences and only this enzyme may be PFA sensitive.

A good correlation between the pK_d, ($-\log_{10}$ dissociation constant of zinc ion-pyrophosphate analogue complex)) of pyrophosphate analogues, as

determined by gel filtration, and their effectiveness as inhibitors of the RNA transcriptase activity of influenza A has been obtained (Cload and Hutchinson, 1983). PFA and PAA have relatively high pK_d' values and are inhibitors of the enzyme (Table 1.3.6.3(ii) and Fig. 1.3.6.3(i)) whereas compounds such as phosphonopropionic acid (PPA) and esters of PAA have lower pK_d' values and are ineffective inhibitors.

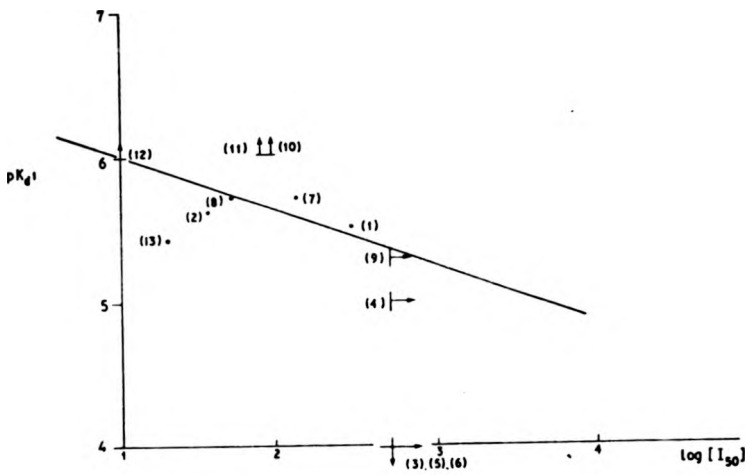
TABLE
INHIBITORY EFFECT OF PYROPHOSPHATE ANALOGUES ON RNA POLYMERASE FROM INFLUENZA VIRUS AND CALF THYMUS DNA POLYMERASE α

	Compound	pK_d'*	Concn. (μM) producing 50% inhibition	
			'flu RNA polymerase	DNA polymerase α
(1)	RCH_2COOH	5.5	275	45
(2)	RCOOH	5.6	35	35
(3)	RCH_2CH_2COOH	< 4	> 500	> 500
(4)	RCHMeCOOH	∿ 5	> 500	> 500
(5)	$RCONH_2$	< 4	> 500	> 500
(6)	$(EtO)_2P(O)CH_2COOH$	< 4	> 500	> 500
(7)	ROR	5.7	125	> 500
(8)	RNHR	5.7	50	> 500
(9)	RCH_2R	5.3	> 500	> 500
(10)	RCHClR	> 6	85	> 500
(11)	$RCCl_2R$	> 6	75	> 500
(12)	$RCBr_2R$	> 6	10	350
(13)	RCOR	5.4	20	100

where R = $(HO)_2P(O)$

*Dissociation constant of complex formed with zinc ions, measured at pH 8.0 as described in text.

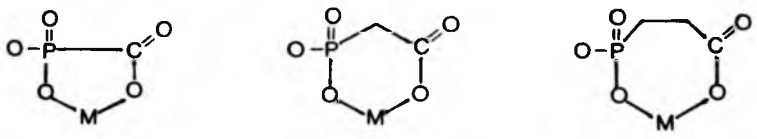
Table 1.3.6.3(ii) Inhibitory effect of pyrophosphate analogues on RNA transcriptase from influenza virus and calf thymus DNA polymerase α. (Cload and Hutchinson, 1983)



I_{50} = Concentration [μM] producing 50% inhibition.

Fig. 1.3.6.3(i) Relationship between pK'_d (pH 8) and effectiveness as inhibitors of the RNA transcriptase activity of influenza A. (Taken from Cload and Hutchinson, 1983.)

The steric factors determining the type of chelate ring formed are likely to be of importance, with PFA forming the most stable 5-membered ring. PAA and PPA would form 6- and 7-membered rings respectively (Fig. 1.3.6.3(ii)) and show progressively less antiviral activity. Particularly active inhibitors were the halogenated methylenebisphosphonates, dibromomethylenebisphosphonate being the most active (50% inhibitor at 10 μM).



(1) (2) (3)

Fig. 1.3.6.3(ii) Metal chelates of (1) PFA, (2) PAA, and (3) PPA.

However, with the exception of carbonyl-bisphosphonate, these compounds showed no antiherpes activity despite forming very stable zinc ion complexes. It appears therefore that much more stringent structural requirements exist for inhibition of HSV DNA polymerase than for influenza RNA transcriptase, which is presumably due to differences in the pyrophosphate binding sites of the two enzymes.

1.3.6.4 Clinical Evaluation

Since PAA exhibits pronounced dermal toxicity and is also deposited in the bone, it has not progressed significantly beyond animal trials (Boezi, 1979). PFA, however, is much less toxic and is currently undergoing clinical trials for topical herpes infections. A clinical study on cutaneous herpes using a 3% (w/w) PFA cream gave some promising results (Öberg, 1983).

Although pyrophosphate analogues accumulate in bones and teeth it is not known whether this results in any deleterious effects (Francis and Centner, 1978).

The design of pyrophosphate analogues with different metal chelating properties should lead to compounds with different effects on bones and which have antiviral activity. One way in which this may be done is to introduce bulky groups into the analogues to make them less compatible with the hydroxy apatite lattice of bones and teeth, or alternatively introduce ligands with a reduced affinity for calcium ions.

1.3.7 Tetrazoles(-CN_4H) as Replacements for Carboxylic Acids (-CO_2H)

Chemical modification of the structure of PAA has not produced a more effective inhibitor of HSV-1 DNA polymerase. However, it has been reported that substitution of the carboxylic acid moiety by the isosteric tetrazole function to give 5-phosphonomethyl-1(H)-tetrazole (1) (Fig. 1.3.7(i)) results in an increase in antiviral activity against HSV-2 in tissue culture but a decrease in activity against HSV-1 (Yaouanc, *et al.*, 1980).

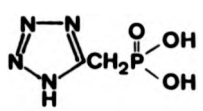
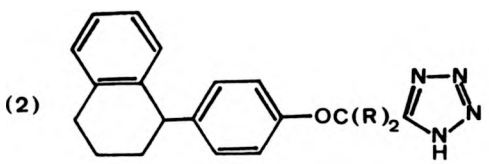


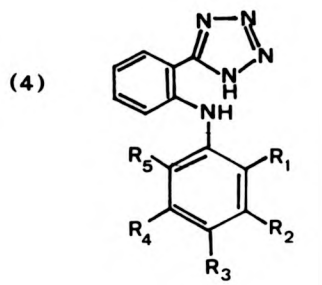
Fig. 1.3.7(i) 5-Phosphonomethyl-1(H)-tetrazole (1).

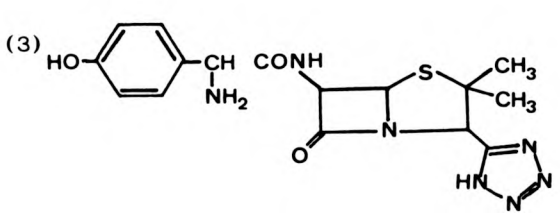
The close similarity between the pKa of 5-substituted tetrazoles and their carboxylic acid analogues has resulted in the synthesis of various biologically active compounds in which this substitution has been carried out. The metabolic stability of the tetrazole function (Epslin and Woodbury, 1956) has also prompted interest in compounds of this type. Of those investigated in recent years, a number of compounds have exhibited improved biological activity, for example in hypocholesterolemic compounds (2) (Fig. 1.3.7(ii)) (Buchanan and Sprancmanis, 1973) and the extended antibacterial activity of the broad-spectrum antibiotic 3-(5-tetrazolyl)penam (3) (Fig. 1.3.7(ii)) (English, *et al.*, 1976). Similar biological activities have been observed for tetrazole analogues of N-phenylanthranilic acid antiinflammatory agents (4) (Fig. 1.3.7(ii)) (Juby, *et al.*, 1968), also pyridyl-substituted 5-(3-pyridyl)tetrazole lipolysis inhibitors (5) (Fig. 1.3.7(ii)) (Holland and Pereira, 1967) with the tetrazole analogue of nicotinic acid (6) being three to four times more potent than nicotinic acid in lowering serum cholesterol in man (Holland and Pereira, 1967).

1.3.7.1 Tetrazole Structure and Properties

The tetrazole ring is a planar 6π-azapyrole type system with two tautomeric forms (a) and (b) (Fig. 1.3.7.1(i)).







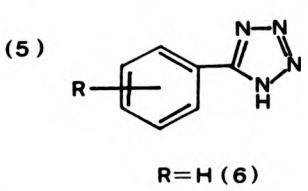


Fig. 1.3.7(ii) Some biologically active tetrazoles in which $-CN_4H$ replaces $-CO_2H$.

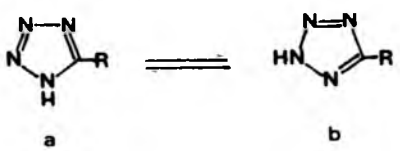


Fig. 1.3.7.1(i)

System (a) is used to number ring substituents throughout this thesis and is known to be the preferred form which dominates the annular tautomerism (Butler, 1977). The tetrazole ring differs from other azole systems in that it represents the functional group for a full series of carbazolic acids (RCN_4H) the full nitrogen analogues of carboxylic acids (RCO_2H).

In addition to those compounds discussed in Section 1.3.7, tetrazole analogues of amino acids are also known and have pKa values that agree closely with those of the corresponding amino acids (McManus and Herbst, 1959). Furthermore, close similarities between the spatial requirements of the tetrazolate and carboxylate ions have been noted, with almost identical anionic charges in similar locations and with similar dimensions (Morley, 1968). Bond lengths are characteristic of an aromatic system, a planar resonance hybrid being indicated by x-ray crystal structure studies. The bond distances and angles of sodium tetrazolate monohydrate are shown in Fig. 1.3.7.1(ii), and sodium formate in (iii).

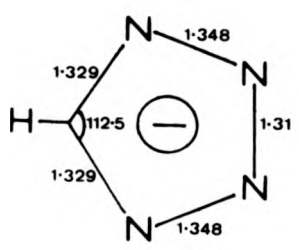
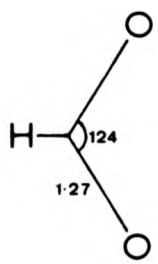


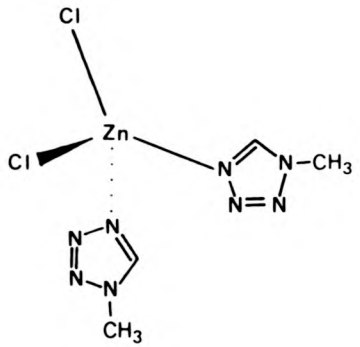
Fig. 1.3.7.1(ii)



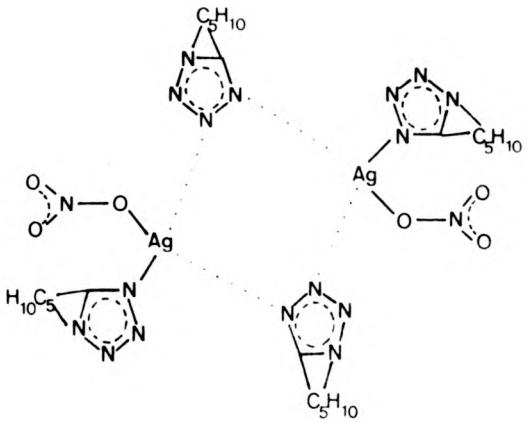
(iii)

Moreover, a wide range of stable metallic complexes of tetrazole systems are known (Butler, 1977) including zinc complexes such as (1) (Baenziger and Schultz, 1971) (Fig. 1.3.7.1(iii)) and complexes with "soft" metal ions such as silver (2) and platinum (3). A distorted tetrahedral complex was reported for dichlorobis (1-methyltetrazolo)zinc(II) (Zn, $(C_2N_4)_2Cl_2$) (1), with monodentate tetrazole ligands which coordinate via a charge-transfer σ-bond between the 4-N atom and the Zn^{2+} which was coplanar with the ring. Complexes of 5-substituted tetrazoles in which tetrazoles behave as monodentate ligands bound at one of the ring nitrogen atoms are known (Weiss and Beck, 1972), and therefore it seems likely that compounds of this type may form metal ion complexes in a similar manner to PAA and other pyrophosphate analogues.

(1)



(2)



(3)

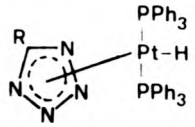


Fig. 1.3.7.1(iii) Metallic complexes of tetrazole systems.

1.4 OUTLINE OF WORK UNDERTAKEN

5-(Phosphonomethyl)-1(H)-tetrazole has been reported as a more effective inhibitor than PAA of HSV-2 in tissue culture, however no studies on influenza viruses have been reported and its metal chelating properties have not been investigated.

Previous studies have shown a correlation between pyrophosphate analogue - zinc ion binding and their effectiveness as inhibitors of the RNA transcriptase activity of influenza virus A.[(Cload,1983)] Most close analogues of PAA and pyrophosphate reported to date have, however, proved to be less active than the parent compounds against herpesviruses. This has made studies on the effect of variation in pyrophosphate analogue-metal ion chelation on these viruses difficult.

The aim of the project was, therefore, the synthesis of 5-(phosphonomethyl)-1(H)-tetrazole and the investigation of its antiviral properties against influenza viruses as well as herpesviruses, and investigation of its metal ion chelating properties, in particular the zinc ion stability constant. Subsequent studies were aimed at the synthesis of some close heterocyclic analogues of 5-(phosphonomethyl)-1(H)-tetrazole which would have different abilities to chelate metal ions by virtue of alterations in the heterocyclic ring structure and its acidity, replacement of phosphono- or carboxylic acid groups by the tetrazole

function and increasing carbon bridge length. Subtle alterations in pyrophosphate analogue structure by replacing certain ligands with others expected to exhibit markedly different behaviour towards certain metal ions was a further target of the synthetic programme.

It was hoped that the study of such compounds would confirm the proposed role of zinc ions in the inhibition of viral DNA polymerase enzymes as well as in the inhibition of RNA transcriptase systems, and could lead to the design of more potent compounds.

It was also of interest to investigate the uptake of pyrophosphate analogues into infected cells, and determine whether any changes occur during viral infection. Differences in the cellular uptake of close analogues of PAA were also of interest with a view to the design of more effective compounds in tissue culture and *in vivo*.

Although there is evidence of varying sensitivities of herpesvirus DNA polymerases to PAA and PFA, there have been no reports on the sensitivities of various strains of influenza virus to pyrophosphate analogues and therefore an investigation into this was proposed.

CHAPTER 2

SYNTHETIC CHEMISTRY

2.1 HETEROCYCLIC COMPOUNDS

The most widely used route to 5-substituted tetrazoles (1) (Fig. 2.1(i)) is by 1,3-dipolar cycloaddition reactions involving addition of azide anion to nitriles.

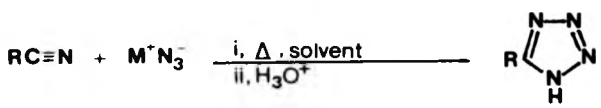


Fig. 2.1(i) Reaction of nitriles and azides to give 5-substituted tetrazoles.

The reaction is, however, highly sensitive to the nature of the cation M^+, the type of nitrile and the solvent used. Reactions using solutions of hydrazoic acid in hydrocarbon solvents (benzene, toluene or xylene) or sodium azide and acetic acid in protic solvents (2-propanol or butanol), require high temperature and pressure and reaction times of up to 7 days (Mihina and Herbst, 1950; Herbst and Wilson, 1957). Dipolar aprotic solvents such as

dimethyl sulphoxide (DMSO) and dimethylformamide (DMF) have been found to facilitate reaction under much milder conditions (Finnegan, *et al.*, 1958). The presence of protic solvents, even in amounts as low as 5% results in large decreases in yields obtained (Finnegan, *et al.*, 1958).

The best sources of anions for reactions in dipolar aprotic solvents are lithium and ammonium salts since they are highly soluble and more easily handled than free hydrazoic acid.

5-Substituted tetrazoles have been prepared in excellent yields using aluminium azide ($Al(N_3)_3$) in refluxing tetrahydrofuran (THF) when NH_4N_3, LiN_3 or NaN_3 in DMF or methoxyethanol have failed (Arnold and Thatcher, 1969). This method is preferred when addition or displacement reactions with azide ion may occur at groups other than the nitrile, resulting in azido intermediates which subsequently decompose. The azide group of $Al(N_3)_3$ does not displace groups such as chloro- from 3-chloropropionitrile, confirming the intramolecular mechanism outlined in Fig. 2.1(ii).

Nitriles bearing electron withdrawing groups react at lower temperatures and with shorter reaction times, although even aliphatic and aromatic nitriles bearing electron-releasing groups undergo 90% or better conversion into tetrazoles in 18-24 hours at 125°C with ammonium or substituted ammonium azides in DMF (Kadaba, 1973). In these conditions Lewis acids

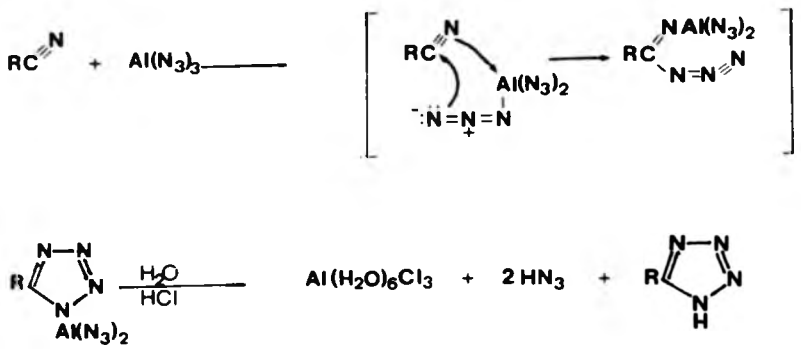


Fig. 2.1(ii) Reaction mechanism for addition of $Al(N_3)_3$ to nitriles.

such as BF_3 have been found to greatly improve yields by coordinating with a nitrile nitrogen and generating a $+\sigma$ charge on the nitrile carbon to facilitate the approach of azide ion.

$$RC{\equiv}N + BF_3 \longrightarrow R\overset{+}{C}{=}N{:}\overset{-}{B}F_3$$

This route (Fig. 2.1(i)) can be used to synthesise a series of 5-5'-bistetrazoles (2) and tetrazole 5-carboxylic acids (3) (Fig. 2.1(iii)).

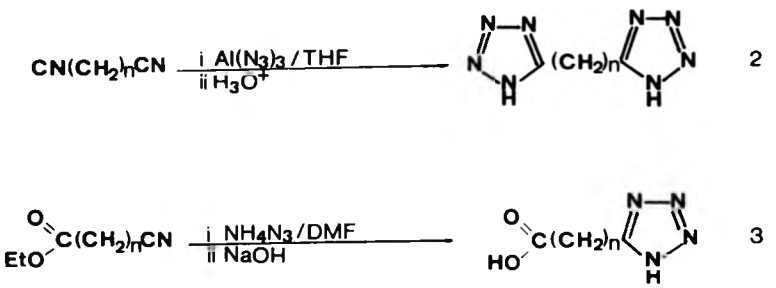


Fig. 2.1(iii) 5-5'-Bistetrazoles (2) and tetrazole 5-carboxylic acids (3).

The synthesis of 5-(phosphonomethyl)-1(H)-tetrazole (4) directly from the corresponding diethyl-cyanomethyl phosphonate and $Al(N_3)_3$ has been reported (Yaouanc, *et al.*, 1980) (Fig. 2.1(iv)).

Fig. 2.1(iv) Synthesis of diethyl 5-(phosphono-methyl)-1(H)-tetrazole (4).

In this study the above reaction was unsuccessful when carried out with $Al(N_3)_3$ in THF,

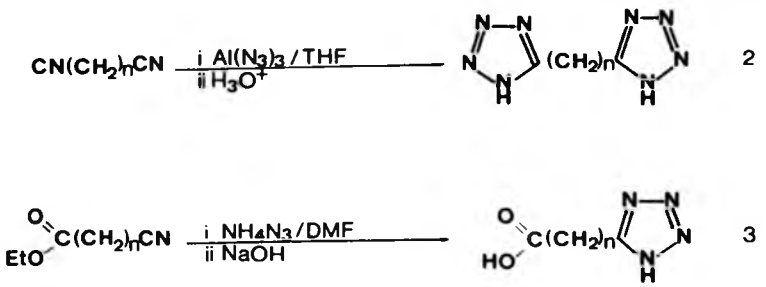


Fig. 2.1(iii) 5-5'-Bistetrazoles (2) and tetrazole 5-carboxylic acids (3).

The synthesis of 5-(phosphonomethyl)-1(H)-tetrazole (4) directly from the corresponding diethyl-cyanomethyl phosphonate and $Al(N_3)_3$ has been reported (Yaouanc, *et al.*, 1980) (Fig. 2.1(iv)).

Fig. 2.1(iv) Synthesis of diethyl 5-(phosphono-methyl)-1(H)-tetrazole (4).

In this study the above reaction was unsuccessful when carried out with $Al(N_3)_3$ in THF,

and also when NH_4N_3 in DMF was employed, even in the presence of BF_3 catalyst. However, in identical conditions chloroalkyl nitriles were found to give yields of 80-90% of the corresponding 5-(chloroalkyl) 1(H)-tetrazoles in relatively short reaction times (Fig. 2.1(v)).

5

Fig. 2.1(v) Synthesis of 5-(chloroalkyl)-1(H)-tetrazoles (5).

5-(Chloroalkyl)-1(H)-tetrazoles can then be converted into the corresponding phosphonates by Michaelis-Arbusov or Michaelis-Becker reactions as described in Section 2.2.

3-(Chloroalkyl)-1,2,4-triazoles can be prepared directly from 1,2,4-triazole (Jones and Ainsworth, 1971) (Fig. 2.1(vi)). Compound (6) can then be converted into its phosphonate by Arbusov or Michaelis-Becker reaction.

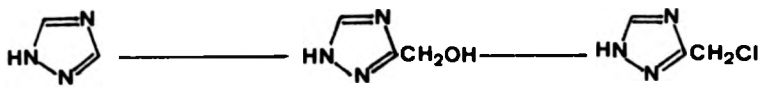


Fig. 2.1(vi) Route to 3-(chloromethyl)-1,2,4-triazole (6).

2.2 SYNTHESIS OF PHOSPHONATES

2.2.1 Pyrophosphate Analogues

Analogues of pyrophosphate such as PAA and methylenebisphosphonate have been prepared by both Arbusov and Michaelis-Becker reactions (Nylen, 1924; Kosolapoff, 1955; Herrin, *et al.*, 1977).

The Michaelis-Arbusov rearrangement or Arbusov reaction (Fig. 2.2.1(i)) is the reaction of an alkyl halide with a trialkyl phosphite, yielding a dialkyl alkylphosphonate (1).

R=alkyl, aryl,etc; R'=alkyl,acyl,etc; hal= Cl, Br, I

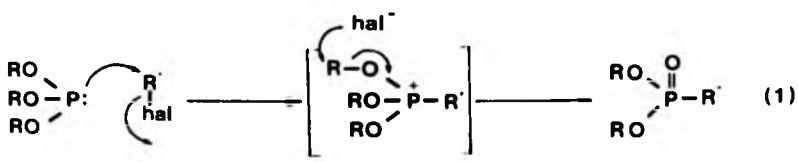


Fig. 2.2.1(i) The Arbusov rearrangement.

The lone pair of electrons of the phosphite attacks the alkyl group of the halide to form the addition compound (1a), followed by dissociation of an alkyl group from (1a), which is unstable in the presence of nucleophiles, to form a phosphonate and a new alkyl halide. The overall result is a conversion of trivalent phosphorus into pentavalent while forming the stable P = O bond.

The Michaelis-Becker reaction involves attack of the sodium salt of a dialkyl phosphite on an alkyl halide to give the phosphonate (1) (Fig. 2.2.1(ii)).

(1)

Fig. 2.2.1(ii) Michaelis-Becker reaction.

In this study the Michaelis-Becker reaction has been used in the synthesis of thiophosphonates (see Chapter 5), while the Arbusov reaction has been used in all other synthetic routes to heterocyclic phosphonates.

The use of 5-(chloroalkyl)-1(H)-tetrazoles in the Arbusov reaction is complicated by the presence of an acidic nitrogen atom on the tetrazole ring.

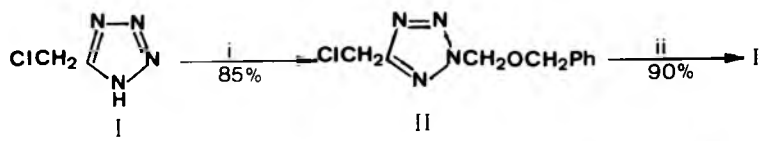
Reaction of unprotected 5-(chloroalkyl)-1(H)-tetrazoles with trialkyl phosphites therefore gives the N-alkylated phosphonate (2) (Fig. 2.2.1(iii)).

Fig. 2.2.1(iii) N-Alkylation of 5-diethylphosphono-alkyl tetrazoles in the Arbusov reaction.

A negative result in the NOE difference spectra of (2) indicates that alkylation occurs preferentially at the 2-position of the tetrazole ring (see Appendix III).

Protection of the tetrazole ring nitrogen is therefore necessary in this reaction. The most satisfactory protecting group has been found to be the N-benzyloxymethyl substituent, which has previously been used to successfully protect pyrole nitrogen atoms (Anderson and Groves, 1971). It can be introduced onto the 5-(chloroalkyl)-1(H)-tetrazole (I) via the sodium salt to afford (II). The group survives the Arbusov reaction conditions and is stable to 4 M hydrochloric acid and refluxing 10% aqueous ethanolic potassium hydroxide (Anderson

Groves, 1971). Removal is achieved in high yield with excess trifluoroacetic acid (TFA) at 20°C (Fig. 2.2.1(iv)).



i NaH/dioxan, 30 min 25°C , $PhCH_2OCH_2Cl-Et_2O$, 12 h 20°C

ii TFA , 72 h 20°C

Fig. 2.2.1(iv) Protection of tetrazole function with the N-benzyloxymethyl group.

The final step in these synthetic routes is the dealkylation of the phosphonate ester produced by the Arbusov reaction. All reactions were carried out with either triethyl phosphite or sodium diethyl phosphite and therefore compounds were diethyl phosphonates. These compounds can be completely de-esterified under vigorous conditions such as refluxing concentrated HCl. Although the tetrazole ring is stable under such conditions isolated yields are generally low.

Bromotrimethylsilane (Me_3SiBr, BTMS) cleanly and quantitatively converts the diethylphosphonates into the corresponding trimethylsilyl phosphonates, which are then readily transformed into the phosphonic acids by hydrolysis with neutral H_2O or methanol

(Fig. 2.2.1(v)).

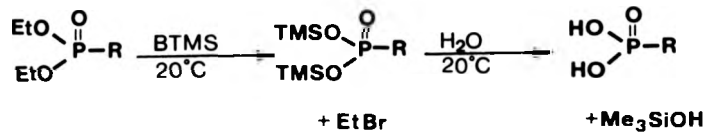


Fig. 2.2.1(v) Dealkylation of diethyl phosphonate esters with BTMS.

Iodotrimethylsiliane (Me_3SiI, ITMS) has also been used to efficiently dealkylate phosphonate esters (Blackburn and Ingleson, 1980). This reagent shows total selectivity for most phosphonate esters but has no effect on aryl phosphate esters or alkyl carboxylate esters and so has been used to prepare C-alkyl esters of PAA and PFA. In this study, ITMS is the preferred reagent for the dealkylation of thiophosphonate esters (see Chapter 5).

2.2.2 Nucleoside-5' Esters of Phosphonates

Nucleoside-5' esters of pyrophosphate analogues can be synthesised using dicyclohexylcarbodiimide (DCC) as condensing agent, with nucleosides protected at the 2' and/or 3' positions (Fig. 2.2.2) as reagents.

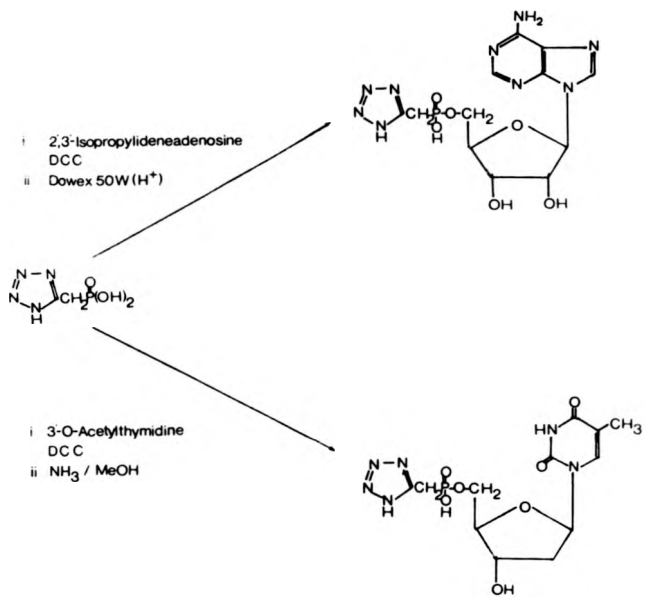
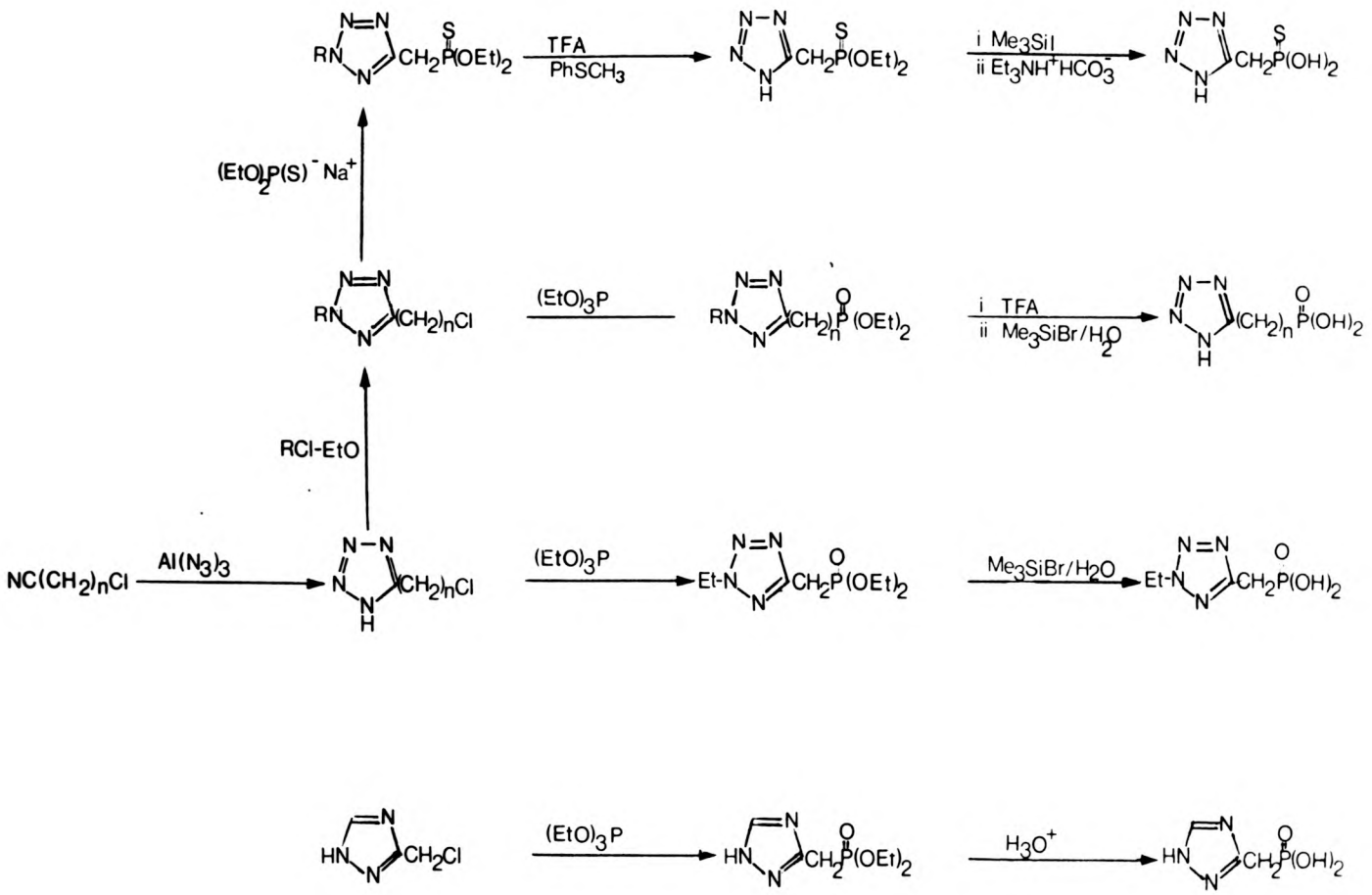


Fig. 2.2.2 Nucleoside-5' esters of 5-phosphonomethyl-1(H)-tetrazole.

The generality and simplicity of this method makes it very popular, although side reactions may result in poor yields.

Because of the poor solubility of tetrazole phosphonates in anhydrous pyridine, the usual solvent for carbodiimide reactions (Gilham and Khorana, 1958), DMF was employed according to Ralph, *et al.* (1963).

Scheme 1 Summary of synthetic routes



R = $PhCH_2OCH_2$

Deprotection of the various hydroxyl functions then gives the corresponding nucleoside-5' phosphonate.

2.3 MATERIALS AND METHODS

2.3.1 Materials

(i) Unless otherwise stated, compounds were commercially available and were used as received (see Appendix I).

(ii) All reagents were of analytical grade or were purified before use.

2.3.2 General Chemical Procedures

(i) ^{1}H n.m.r. spectra were recorded at 220 MHz using a Perkin-Elmer R34 spectrometer, and chemical shifts are quoted with either tetramethylsilane (TMS) (in $CDCl_3$ or d_6-DMSO) or sodium 3-(trimethylsilyl)-1-propane sulphonate (TSS) (in D_2O) as standards. ^{31}P n.m.r. spectra were recorded at 36.44 MHz using a Bruker WH90 spectrometer and were proton decoupled. ^{31}P n.m.r. shifts are quoted relative to an external standard (85% H_3PO_4) with downfield shifts positive.

(ii) Chemical ionisation mass spectra were determined using ammonia as reagent gas as previously described (Cload and Hutchinson, 1983).

(iii) Chromatography was performed by upward development for t.l.c. plates and downward development for paper, using the solvent systems described in (iv)

for silica t.l.c., all other systems are described below:

System	Plate/Paper	Solvent
I	Cellulose F_{254}/W3MM	BuOH/AcOH/H_2O (5:2:3)
II	Cellulose F_{254}	EtOH/0.5 M NH_4OAc (5:2)
III	Whatman 3MM	PrOH/NH_4OH/H_2O (7:1:2)
IV	Whatman 3MM	MeOH/NH_4OH/H_2O (6:1:3)

Phosphonates and their nucleoside-5' esters were visualised on descending paper chromatograms and cellulose t.l.c. plates by the method of Ames and Bochner (1981) for the detection of phosphate esters on chromatograms. Silica t.l.c. spots were visualised by fluorescence under 254 nm light and/or iodine vapour.

(iv) Triazoles, tetrazoles and their phosphonate esters were purified by silica gel column chromatography by the method of Still (1978) using the following solvent systems:

System	Solvent(s)
A	Acetone/60-80^{o} petroleum ether (3:7, v/v)
B	$CHCl_3$
C	Methanol/$CHCl_3$ (1:9, v/v)
D	Ethyl acetate

Phosphonates and their nucleoside-5' esters were purified by either DEAE-Sephadex A-25 or DEAE-cellulose DE52 ion-exchange columns eluted with triethylammonium bicarbonate buffer as described in Section 2.4.

(v) Elemental analyses were carried out by Elemental Micro-Analysis Limited, Okehampton, Devon, U.K., CHN Limited, Leicester, U.K., and Butterworth Laboratories Limited, Teddington, Middlesex, U.K.

2.4 EXPERIMENTAL

2.4.1 5-(Phosphonomethyl)-1(H)-Tetrazole (1)

5-(Chloromethyl)-1(H)-tetrazole was prepared by heating chloroacetonitrile (6.4 ml, 0.1 mol) with dry sodium azide (29.5 g, 0.44 mol) and anhydrous aluminium chloride (13.3 g, 0.1 mol) in dry THF (200 ml) under reflux in a nitrogen atmosphere for 14 hours. The reaction mixture was cooled, acidified with 5 M HCl and then extracted with ethyl acetate (3 x 100 ml). The combined THF and ethyl acetate extracts were dried (over Na_2SO_4) and the solvent removed *in vacuo*. The residue was recrystallised from chloroform to give the chloromethyl tetrazole (9.6 g, 81%), m.p. 85°, lit. 85°, ^1H n.m.r. (d_6 DMSO) δ 5.1 p.p.m., CIMS m/z 119 $[M + H]^+$, 136 $[M + NH_4]^+$.

2-(Benzyloxymethyl)-5-(chloromethyl)-tetrazole was prepared by adding to the tetrazole obtained above

(5.0 g, 1 equiv.) in dry dioxan (60 ml), with stirring, sodium hydride (50% suspension in oil, 2.25 g, 1.1 equiv.). After 30 minutes, benzyl chloromethyl ether (6.0 g, 1 equiv.) was added and the mixture stirred for 16 hours at 25°C. The reaction mixture was then filtered, the solvent removed from the filtrate *in vacuo* and the residue purified by silica gel column chromatography (solvent A) to give 2-(benzyloxymethyl)-5-(chloromethyl)-tetrazole (8.6 g, 85%), ^{1}H n.m.r. ($CDCl_3$) δ 4.65 (2H, s), 4.8 (2H, s), 5.9 (2H, s) and 7.3 p.p.m. (5H, m), CIMS m/z 239 $[M + H]^+$, 256 $[M + NH_4]^+$.

2-(Benzyloxymethyl)-5-(chloromethyl)-tetrazole (2.4 g, 0.01 mol) was stirred at 120°C for 14 hours in triethyl phosphite (5.0 g, 0.033 mol). After this time, excess triethyl phosphite was removed *in vacuo* and the residue purified by silica chromatography (solvent B) to give the diethyl ester of 2-(benzyloxymethyl)-5-(phosphonomethyl)-tetrazole (2.2 g, 65%), ^{1}H n.m.r. ($CDCl_3$) δ 1.3 (6H, t, J = 6 Hz), 3.55 (2H, d, $^2J_{P-H}$ = 20 Hz), 4.2 (4H, m), 4.65 (2H, s), 5.9 (2H, s) and 7.3 p.p.m. (5H, m), CIMS m/z 341 $[M + H]^+$, 358 $[M + NH_4]^+$.

This diethyl ester was stirred in trifluoroacetic acid (8 ml) for 72 hours at room temperature. Removal of the solvent *in vacuo* afforded diethyl 5-(phosphonomethyl)-1(H)-tetrazole (1.3 g, 90%) after purification by silica chromatography (solvent C), ^{1}H n.m.r. ($CDCl_3$) δ 1.35 (6H, t, J = 6 Hz), 3.75 (2H, d, $^2J_{P-H}$ = 20 Hz) and 4.25 p.p.m. (4H, m,

J = 7 Hz), CIMS m/z 221 $[M + H]^+$, 238 $[M + NH_4]^+$.

Diethyl 5-(phosphonomethyl)-1(H)-tetrazole (1.1 g, 0.005 mol) was stirred for 2 hours at room temperature with bromotrimethylsilane (1.53 g, 0.01 mol). Evaporation of the silane gave a residue to which excess water was added, the latter was removed by lyophilisation and the addition of water and lyophilisation repeated several times. The product was purified by ion-exchange on DEAE-Sephadex A25 (Et_3NH^+ form, 1.5 x 45 cm) and elution with a gradient of 0.5-1.0 M triethylammonium bicarbonate (pH 8.4) to give the triethylammonium salt of 5-(phosphonomethyl)-1(H)-tetrazole which was converted using a Dowex 50 (H^+ form) ion-exchange column, into the free acid (615 mg, 75%), m.p. 170° (dec.), ^1H n.m.r. (D_2O) δ 3.5 p.p.m. (d, $^2J_{P-H}$ = 20 Hz), ^{31}P n.m.r. (D_2O) δ 16.9 p.p.m., analysis C, 14.6; H, 3.33; N, 33.12; P, 18.42%; $C_2H_5N_4O_3P$ requires C, 14.64; H, 3.07; N, 34.15; P, 18.88%.

2.4.2 5-(2-Phosphonoethyl)-1(H)-tetrazole (2)

5-(2-Chloroethyl)-1(H)-tetrazole was prepared as described above, from 3-chloropropionitrile, in yield of 70% (m.p. 103°, lit. 103-104°) after recrystallisation from water.

5-(2-Chloroethyl)-1(H)-tetrazole was N-protected as described above and purified by silica chromatography (solvent A) to afford 2-(benzyloxymethyl)-5-(chloroethyl)-tetrazole (75%), ^1H n.m.r.

($CDCl_3$) δ 3.35 (2H, t, J = 7 Hz), 3.9 (2H, t, J = 7 Hz), 4.6 (2H, s), 5.85 (2H, s) and 7.3 p.p.m. (5H, m), CIMS m/z 253 $[M + H]^+$, 270 $[M + NH_4]^+$.

2-(Benzyloxymethyl)-5-(chloroethyl)-tetrazole (2.5 g, 0.01 mol) was stirred at 120°C for 24 hours in triethyl phosphite (5.0 g, 0.033 mol). Excess triethyl phosphite was removed *in vacuo* and the residue purified by silica chromatography (solvent B) to give the diethyl ester of 2-(benzyloxymethyl)-5-(phosphonoethyl)-tetrazole (2.1 g, 60%), ^1H n.m.r. ($CDCl_3$) δ 1.3 (6H, t, J = 6 Hz), 2.3 (2H, m), 3.2 (2H, m), 4.1 (4H, m), 4.6 (2H, s), 5.85 (2H, s), and 7.3 p.p.m. (5H, m), CIMS m/z 355 $[M + H]^+$.

Deprotection was carried out as described above to afford diethyl 5-(phosphonoethyl)-1(H)-tetrazole (1.2 g, 85%) after purification by silica column (solvent C), ^1H n.m.r. ($CDCl_3$) δ 1.3 (6H, t, J = 6 Hz), 2.3 (2H, m), 3.35 (2H, m) and 4.15 p.p.m. (4H, m), CIMS m/z 235 $[M + H]^+$, 252 $[M + NH_4]^+$.

Dealkylation with bromotrimethylsilane as described, followed by purification on DEAE-cellulose DE52 (Et_3NH^+ form, 2.5 x 45 cm) ion-exchange column eluted with 0.5-1.0 M triethylammonium bicarbonate (pH 8.4) gave the triethylammonium salt of 5-(phosphonoethyl)-1(H)-tetrazole. Conversion into the sodium salt by Dowex 50 (Na^+ form) column afforded 5-(2-phosphonoethyl)-1(H)-tetrazole as the disodium salt (774 mg, 68%), ^1H n.m.r. (D_2O) δ 2.1 (2H, m), 3.2 p.p.m. (2H, m),

^{31}P n.m.r. (D_2O) δ 24.7 p.p.m., analysis C, 15.97; H, 3.11; N, 26.21; P, 13.88%; $C_3H_5N_4O_3PNa_2$ requires C, 16.20; H, 2.3; N, 25.20; P, 13.95%.

2.4.3 2-Ethyl-5-(phosphonomethyl)-tetrazole (3)

5-(Chloromethyl)-1(H)-tetrazole (1.2 g, 0.01 mol) was heated in triethyl phosphite (5.0 g, 0.03 mol) at 120°C for 14 hours. Excess triethyl phosphite was then removed *in vacuo* and the residue purified by silica chromatography (solvent A) to give diethyl 2-ethyl-5-(phosphonomethyl)-tetrazole (1.9 g, 80%), ^1H n.m.r. ($CDCl_3$) δ 1.2 (6H, t, J = 6 Hz), 1.5 (3H, t, J = 6 Hz), 3.4 (2H, d, $^2J_{P-H}$ = 20 Hz), 4.1 (4H, m, J = 7 Hz) and 4.55 p.p.m. (2H, q, J = 7 Hz), CIMS m/z 249 $[M + H]^+$, 266 $[M + NH_4]^+$.

Diethyl 2-ethyl-5-(phosphonomethyl)-tetrazole (1.0 g, 0.004 mol) was stirred at room temperature for 2 hours with bromotrimethylsilane (1.5 g, 0.01 mol). Evaporation of the silane *in vacuo* gave a residue to which excess water was added. The latter was removed by repeated lyophilisation to leave a product which was purified by chromatography on a DEAE-Sephadex A25 column (1.5 x 45 cm) and elution with a gradient of 0-0.5 M triethylammonium bicarbonate (pH 8.4) to give the triethylammonium salt of 2-ethyl-5-(phosphonomethyl)-tetrazole which was converted into the free acid using a Dowex 50 (H^+ form) column, yield 320 mg (41%), ^1H n.m.r. (D_2O) δ 1.45 (3H, t, J = 6 Hz), 3.4 (2H, d,

$^{2}J_{P-H}$ = 20 Hz), 4.6 p.p.m. (2H, q, J = 7 Hz), ^{31}P n.m.r. (D_2O) δ 18.8 p.p.m., analysis C, 23.6; H, 5.02; N, 26.7; P, 14.79%; $C_4H_9N_4O_3P.H_2O$ requires C, 22.86; H, 5.20; N, 26.67; P, 14.76%.

2.4.4 3-(Phosphonomethyl)-1,2,4-triazole (4)

3-(Chloromethyl)-1,2,4-triazole (3.0 g, 0.025 mol) which had been prepared as described (Jones and Ainsworth, 1971), was heated with triethyl phosphite (12.5 g, 0.075 mol) at 120°C for 12 hours. Excess triethyl phosphite was removed *in vacuo* and the residue purified by silica chromatography (solvent C) to afford diethyl 3-(phosphonomethyl)-1,2,4-triazole (2.1 g, 35%), ^{1}H n.m.r. ($CDCl_3$) δ 1.3 (6H, t, J = 6 Hz), 3.55 (2H, d, $^{2}J_{P-H}$ = 20 Hz), 4.2 (4H, m, J = 7 Hz), 8.3 p.p.m. (1H, s), CIMS m/z 220 $[M + H]^+$.

The diethyl ester (1.5 g, 0.007 mol) was heated under reflux in concentrated HCl (10 ml) for 12 hours, the excess HCl was then removed *in vacuo* and the residue purified by chromatography on DEAE-cellulose DE52 column (2.5 x 50 cm, Et_3NH^+ form) with elution by a gradient of 0-0.5 M triethylammonium bicarbonate (pH 8.4) to give triethylammonium 3-(phosphonomethyl)-1,2,4-triazole. Using a Dowex 50 (Na^+ form) column this was converted into the disodium salt (700 mg, 44%), ^{1}H n.m.r. (D_2O), δ 3.3 (2H, d, $^{2}J_{P-H}$ = 20 Hz), 8.3 p.p.m. (1H, s), ^{31}P n.m.r. (D_2O) δ 17.4 p.p.m., analysis C, 15.37; H, 4.2; N, 19.6; P, 13.5;

$C_3H_3N_3O_3PNa_2.H_2O$ requires C, 16.0; N, 2.67; N, 18.67; P, 13.8%.

2.4.5 Adenosine-5'-(5-phosphonomethyl)-1(H)-Tetrazole (9)

To a solution of the anhydrous pyridinium salt of 5-(phosphonomethyl)-1(H)-tetrazole (150 mg, 0.6 mmol) and 2',3'-*O*-isopropylidene adenosine (184 mg, 0.6 mmol) in anhydrous DMF (4 ml) was added a solution of dicyclohexylcarbodiimide (600 mg, 3 mmol) in DMF (3 ml). The mixture was stirred at 20°C for 96 hours, water (5 ml) was added and the stirring continued for a further 24 hours. The reaction mixture was then filtered and the filtrate evaporated to dryness *in vacuo*. The residue was then purified by chromatography on a DEAE-cellulose (Et_3NH^+ form, 2.5 x 40 cm) column with elution by a gradient of 0-0.5 M triethylammonium bicarbonate (pH 8.2), after following the reaction by t.l.c. (solvent systems II and III). 2',3'-*O*-isopropylidene adenosine-5'-(5-phosphonomethyl)-1(H)-tetrazole was isolated as the triethylammonium salt (90 mg, 25%) after monitoring the eluate by u.v. absorption (254 nm) (R_f (II) = 0.24, R_f (III) = 0.57), ^1H n.m.r. (D_2O), δ 1.3 (18H, t, J = 6.5 Hz), 1.6 (3H, s), 1.8 (3H, s), 3.25 (12H, q, J = 7 Hz), 3.45 (2H, d, $^2J_{P-H}$ = 20 Hz), 4.15 (2H, m), 4.7 (1H, m), 5.15 (1H, m), 5.3 (1H, m), 6.3 (1H, d, J = 4 Hz), 8.3 (1H, s), 8.45 p.p.m. (1H, s).

Removal of the isopropylidene group and

isolation of the free acid of (5) was achieved by passing the triethylammonium salt down a Dowex 50 (H^+ form, 1.5 x 12 cm) ion-exchange column to give adenosine-5'-(5-phosphonomethyl)-1H-tetrazole (52 mg, 18%), chromatography, R_f (II) = 0.1, R_f (III) = 0.37, ^1H n.m.r. (D_2O) δ 3.5 (2H, d, $^2J_{P-H}$ = 20 Hz), 4.3 (2H, m), 4.5 (1H, m), 4.6 (1H, m), 5.1 (1H, m), 6.3 (1H, d, J = 4 Hz), 8.5 (1H, s), 8.6 p.p.m. (1H, s), UV_{max} pH 7.0 259 nm (ε 16 400), analysis C, 34.9; H, 4.63; N, 27.0; P, 6.8%; $C_{12}H_{16}N_9O_6P.4H_2O$ requires C, 32.0; H, 5.7; N, 27.8; P, 6.2%.

2.4.6 Thymidine-5'-(5-phosphonomethyl)-1(H)-Tetrazole (10)

The 3'-*O*-acetyl thymidine-5' ester of 5-(phosphonomethyl)- 1(H)-tetrazole was prepared in 50% yield as the triethylammonium salt from 3'-*O*-acetyl-thymidine and (1) as described above. The acetyl group was removed by treating this salt (380 mg, 0.7 mmol) with methanol (10 ml) which had been saturated with gaseous ammonia. The product of this reaction, after removal of excess methanol, was purified by chromatography on a DEAE-cellulose column (Et_3NH^+ form, 2.5 x 45 cm) with elution by gradient of 0-0.5 M triethylammonium bicarbonate (pH 8.4) and finally converted into the free acid with a Dowex 50 (H^+ form, 1.5 x 12 cm) column to afford thymidine-5'-(5-phosphonomethyl)-1(H)-tetrazole (140 mg, 54%), R_f (III) = 0.42, R_f (IV) = 0.63,

^1H n.m.r. (D_2O), δ 1.7 (3H, s), 2.3 (2H, m), 3.45 (2H, d, $^2J_{P-H}$ = 20 Hz), 4.1 (3H, m), 4.5 (1H, m), 6.25 (1H, m), 7.5 p.p.m. (1H, s), U V $_{max}$ pH 7.0 266 nm (ε 12 800), analysis C, 35.10; H, 4.45; N, 20.65; P, 8.19%; $C_{12}H_{17}N_6O_7P.H_2O$ requires C, 35.47; H, 4.46; N, 20.68; P, 7.62%.

2.4.7 5,5'-Methylenebis-1(H)-tetrazole (5)

To a solution of malononitrile (2.2 g, 0.03 mol) in dry THF (150 ml) was added dry sodium azide (20.0 g, 0.3 mol) with stirring, followed by dry, powdered aluminium trichloride (9.0 g, 0.03 mol). The mixture was heated for 14 hours under reflux in a nitrogen atmosphere, then cooled and acidified with 5 M HCl. The solution was extracted with ethyl acetate (3 x 100 ml), the combined extracts dried (Na_2SO_4) and the solvent removed *in vacuo*. The residue was recrystallised from acetonitrile to give 5,5'-methylenebis-1(H)-tetrazole (2.3 g, 50%) m.p. 208-210°(dec.), lit. 215°, ^1H n.m.r. (D_2O), δ 4.7 p.p.m. (2H, s), CIMS m/z 153 $[M + H]^+$, 170 $[M + NH_4]^+$, analysis C, 23.84; H, 2.33; N, 73.79%; $C_3H_4N_8$ requires C, 23.70, H, 2.65; N, 73.66%.

2.4.8 5,5'-Ethylenebis-1(H)-tetrazole (6)

5,5'-Ethylenebis-1(H)-tetrazole was prepared in an analogous manner from succinonitrile (8.0 g, 0.1 mol) to give 9.7 g, (60%) product, m.p. 234°(dec.),

lit 230° after recrystallisation from water, ^1H n.m.r. (D_2O), δ 3.5 p.p.m. (4H, s), CIMS m/z = 167 $[M + H]^+$, 184 $[M + NH_4]^+$, analysis C, 28.90; H, 3.22; N, 64.1%; $C_4H_6N_8$ requires C, 28.90; H, 3.64; N, 64.1%.

2.4.9 1(H)-Tetrazole-5-ethanoic Acid (7)

A solution in DMF (100 ml) of ethyl cyanoacetate (11.5 g, 0.1 mol), ammonium chloride (5.9 g, 0.11 mol) and dry sodium azide (7.15 g, 0.11 mol) was heated at 100°C for 16 hours under dry nitrogen. Excess DMF was removed *in vacuo*, the residue dissolved in water (50 ml) and acidified to pH 2.0 with 5 M HCl. This mixture was cooled to 5°C whereupon crystals separated out which were filtered off and washed with ice-water. Recrystallisation from propan-2-ol gave ethyl 1(H)-tetrazole-5-ethanoate (7.6 g, 50%), m.p. 128°, lit. 128-130°.

The ethyl ester (500 mg, 3.2 mmol) was heated under reflux in 3 M NaOH solution (20 ml) for 4 hours. This solution was neutralised with 5 M HCl and evaporated to dryness *in vacuo*. The residue was extracted with hot acetonitrile (10 ml), filtered and the acetonitrile evaporated to dryness. The 1(H)-tetrazole-5-ethanoic acid obtained was recrystallised from acetonitrile, yield 360 mg (93%), m.p. 170°, ^1H n.m.r. (D_2O), δ 4.1 p.p.m. (2H, s), CIMS m/z 129 $[M + H]^+$, 146 $[M + NH_4]^+$, analysis C, 28.17; H, 3.10, N, 43.83%; $C_3H_4N_4O_2$ requires C, 28.13; H, 3.15;

N, 43.74%.

2.4.10 1(H)-Tetrazole-5-(3'-propionic Acid) (8)

5-(2-Cyanoethyl)-1(H)-tetrazole was a by-product formed in the synthesis of (6) and was purified by silica chromatography (solvent D), m.p. 140-142°, ^{1}H n.m.r. (d_6-DMSO) δ 3.55 (2H, t, J = 6.5 Hz), 3.8 p.p.m. (2H, t, J = 6.5 Hz), CIMS m/z 124 $[M + H]^+$.

5-(2-Cyanoethyl)-1(H)-tetrazole (50 mg, 0.4 mmol) was heated under reflux in solution in 20% HCl/glacial acetic acid (2.5 ml, 1:1 v/v) for 12 hours. Excess acid was removed *in vacuo* and the residue recrystallised from propan-2-ol to give 1(H)-tetrazole-5-(3'-propionic acid) (36 mg, 62%) m.p. 218-220° (dec.), ^{1}H n.m.r. (D_2O), δ 2.8 (2H, t, J = 6.5 Hz), 3.22 p.p.m. (2H, t, J = 6.5 Hz), analysis C, 33.8; H, 4.20; N, 38.91%; $C_4H_6N_4O_2$ requires C, 33.8; H, 4.25; N, 39.42%.

CHAPTER 3

HERPESVIRUSES

3.1 METHODS

3.1.1 Virus Stocks

The KOS and HFEM strains of HSV-1 and the 3345 strain of HSV-2 were employed, which had been maintained in the laboratories of Roche Products Ltd., Welwyn Garden City, by low multiplicity passage in Vero cells. The PAA resistant strain (PAA^r) was HSV-1 18 Clone I (Honess and Watson, 1977) originally supplied to Roche Products Ltd., by R. Honness, N.I.M.R., Mill Hill, London.

3.1.2 Cell Culture and Media

HeLa (BU25) cells were used for the preparation of viral DNA polymerase. Infectivity assays were carried out using serially propagated African green monkey kidney (Vero) cells. HeLa (BU25) cell cultures were grown as monolayers in roller bottles (2.5 L) at 37°C in growth medium (~ 150 ml of HEPES-buffered Eagle's minimal essential medium (EMEM) supplemented with non-essential amino acids (NEAA, 1%), foetal calf serum (FCS, 10%), L-glutamine (4 mM), penicillin (100 units/ml) and streptomycin (100 µg/ml). The concentration of FCS was reduced to 1% for maintenance of cells. Vero cells

were grown as monolayer cultures in flat culture bottles (175 ml) at 37°C in medium (~ 100 ml) of HEPES-buffered Dulbecco's modification of Eagle's medium supplemented with newborn calf serum (NCS, 10%), L-glutamine (4 mM), penicillin (100 units/ml) and streptomycin (100 µg/ml). For maintenance of cells 1% NCS was employed. In all cases cells were passaged using a trypsin (0.05%), EDTA (0.02%) HEPES-buffered balanced salt solution.

3.1.3 Plaque Reduction Assay

Confluent 175 ml flat culture bottles of Vero cells were used, trypsinised and resuspended in growth medium (5 ml) and pooled together for a viable cell count. Cells were diluted to 2 x 10^5 cells/ml medium and 24-well plates inoculated with cell suspension (1 ml per well). Growth medium was removed after 24 hours at 37°C when the cells were confluent monolayers and the cells infected with freshly diluted virus in maintenance medium (50 p.f.u. in 0.2 ml). After virus absorption (1 hour, 4°C) cells were overlaid with 0.7 ml of maintenance medium containing 1% carboxymethyl cellulose (CMC) followed by drug dilutions (0.1 ml, in maintenance medium) in triplicate wells. Cells were incubated for 3-4 days at 37°C in a humidified atmosphere of carbon dioxide (5%) in air. Cells were then fixed with formal saline solution (FSS) (~ 3 ml, 4% (v/v) formaldehyde in phosphate buffered

saline (PBS)/well) for 15-30 minutes. FSS was removed and plates stained with Giemsa, washed with water and dried. Plaques were counted and mean results used to plot semi-logarithmic dose-response curves, from which the drug concentrations required to reduce the virus control figure by 50% (ID_{50}) were determined. Drug control wells, containing no virus, were used to observe any drug-induced cellular cytotoxicity.

3.1.4 Induction of DNA Polymerase Activity in HSV-Infected Cells

The procedure used was a modification of that of Powell and Purifoy (1977) and Cload (1983). Confluent monolayers of HeLa (BU25) cells in roller bottles (approx. 10^8 cells/roller bottle) were infected with HSV-1 (KOS) at a m.o.i. of 2-10 p.f.u./cell in maintenance medium (10 ml, EMEM plus supplements) and incubated for 90 minutes at 37°C. At the end of the period of absorption fresh medium was added (1% FCS, 50 ml) and the previous medium and unabsorbed virus discarded. The cell monolayers were incubated for 16 hours at 37°C, when cytopathic effect was beginning to be observed. Monolayers were then scraped into the medium and pelleted by low speed centrifugation (2000 r.p.m./15 minutes/4°C). Cells were resuspended in cold PBS (40 mls) and centrifuged (2000 r.p.m./15 minutes/4°C), the supernatant discarded and the process repeated twice. Cells were resuspended in cold buffer (10 mM *tris*-HCl, pH 7.5, 3 mM 2-mercaptoethanol) at a concentration of 10^7

cells/ml. The cells were then sonicated (1 minute/ full power, MSE soniprobe). An equal volume of high salt buffer (3.4 M NaCl, 10 mM *tris*-HCl, 3 mM 2-mercapto-ethanol, pH 7.5) was then added and the suspension left for 40 minutes at 4°C. DNA and protein precipitates were removed by centrifugation (30 000 g/20 minutes), and the supernatant dialysed for 3 hours against buffer (500 ml, 10 mM *tris*-HCl, pH 7.5, 3 mM 2-mercaptoethanol, 10% (v/v) glycerol). The dialysate was then centrifuged again at 30 000 g/15 minutes before dialysing for 3 hours (500 ml buffer) followed by overnight dialysis (1 L buffer). The dialysate was clarified by centrifugation (100 000 g/1 hour) and stored in 1.5 ml aliquots at -70°C and thawed once prior to use.

3.1.5 DNA Polymerase Assays

HSV DNA polymerase activity was assayed in a 200 µl reaction mixture containing 50 mM *tris*-HCl buffer (pH 7.8), 5 mM $MgCl_2$, 20 mM KCl, 110 mM $(NH_4)_2SO_4$, 200 µM EDTA, 6 mM 2-mercaptoethanol, 25 µM each of dATP, dCTP, dGTP, 6 µM [methyl-^{3}H]-TTP (1000-2000 cpm/pmol), and 375 µg/ml DNase I treated salmon sperm DNA (prepared by the method of Cload (1983)) and DNA polymerase (20 µl of enzyme incorporated about 25 pmol dTMP per 200 µl reaction mixture in 30 minutes).

Calf thymus DNA polymerase activity was assayed in a 200 µl reaction mixture containing 50 mM *tris*-HCl buffer (pH 7.0), 1 mM dithiothreitol, 500 µg/ml

BSA, 10 mM $MgCl_2$, 20 mM KCl, 200 µg/ml DNase I treated salmon sperm DNA, 20 µM [^{3}H]-dTTP (400-1000 cpm/pmol), 100 µM each of dATP, dCTP, dGTP and enzyme (0.01 unit).

Reaction mixes were kept at 0°C until addition of enzyme, and were then incubated at 37°C for 30 minutes (HSV-DNA polymerases) or 60 minutes (calf thymus DNA polymerase α) during which time a linear increase in incorporation of radioactivity into acid-precipitable material was obtained. Reactions were stopped by the addition of 200 µl TCA (20%, w/v) and the mixtures kept on ice for 12 hours. Acid-insoluble material was collected by suction filtration onto GF/C discs (Whatman) wetted with cold TCA (10%, w/v), on a Millipore 1225 sampling manifold. Discs were washed with 1 x 10 ml cold 10% (w/v) TCA, 2 x 10 ml cold 5% (w/v) TCA, 1 x 10 ml ethanol and then oven dried for 15 minutes at 80°C before counting in a toluene based scintillant. Zero-time control tubes were obtained by adding 200 µl 10% (w/v) TCA before addition of polymerase. ID_{50} values were obtained from dose-response curves for each compound tested.

3.1.6 Determination of Heterocyclic Pyrophosphate Analogue - Zinc Ion Stability Constants

The zinc ion stability constants for each compound were determined by the method of Hummel and Dreyer (1962) by a gel-filtration method using a

column of Sephadex G-10 (1.6 x 92 cm) equilibrated with zinc chloride (10 μM, Sepctroscopic grade) in buffer (0.1 M triethanolamine-HCl, pH 8.0). The pyrophosphate analogues (150 or 200 nmoles) were added in buffer (1.5 ml) and the column eluted with the same buffer at a flow rate of 0.4 ml/minute. The zinc content of 2.5 ml fractions was determined by atomic absorption spectrometry and the stability constants determined as described by Hummel and Dreyer (1962). Stability constants are represented by the term $pK_{d'}$, defined here as $-\log_{10}$ (dissociation constant of zinc ion-pyrophosphate analogue complex).

A description of the calculation of K_d values is given in Appendix II (reprint of N. Yoza, *J. Chem. Ed.*, 54, 247 (1977).

3.1.7 Uptake of Phosphonoacetic Acid and 5-(Phosphonomethyl)-1(H)-tetrazole (1) by HSV-Infected and Uninfected Cells

3.1.7.1 [2-^{3}H]-Phosphonoacetic Acid and [2-^{3}H]-5-(Phosphonomethyl)-1(H)-tetrazole

To anhydrous PAA or (1) (10 mg) dissolved in carrier free ^3H_2O (50 μl) was added concentrated HCl (2 μl) and the mixture heated at 70°C for 5 hours. Excess ^3H_2O was removed *in vacuo* followed by repeated addition and removal of unlabelled water and lyophilisation. [2-^{3}H]-PAA thus obtained had a specific activity of 9.2 μCi/μmole, [2-^{3}H]-(1) had a specific activity of 7.2 μCi/μmole, both compounds co-chromatographed

with unlabelled materials in solvent I.

3.1.7.2 Uptake of [2-^{3}H]-PAA and (1) into HSV-Infected and Uninfected Vero Cells

Vero cells were grown to confluency in 35 mm culture dishes (10^6 cells/dish). The medium was removed and the cell monolayers infected with 10^7 p.f.u./dish (5 p.f.u./cell) of freshly diluted virus (HSV-1 18 CI, PAA^r) in medium (200 µl) or mock infected with 200 µl medium. After 1 hour at 4^{o}C medium was removed and cell monolayers washed once with maintenance medium (1.5 ml) before a known activity and concentration of [2-^{3}H]-PAA or [2-^{3}H]-(1) was added, this was 5 µCi/ml in 1.0 ml medium per plate in experiments A and B, and 0.5 µmol in experiment C.

Dishes were incubated at 37^{o}C in an atmosphere of CO_2 (5%) in air for times of up to 24 hours. After the desired incubation period the medium was decanted and the cell monolayers washed with 3 x 1 ml PBS, followed by solubilisation with 3 M KOH (300 µl, incubated at 37^{o}C for 1 hour). Solubilised cells were neutralised with 6 N HCl before counting 350 µl aliquots in 10 mls scintillant. Early cytopathic effect was observed microscopically after 24 hours when using virus infected at 5 p.f.u./cell, at higher multiplicities of infection cells were lost at this stage resulting in low counts.

3.2 EXPERIMENTAL AND RESULTS

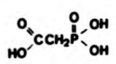
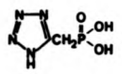
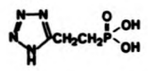
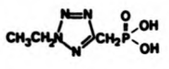
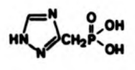
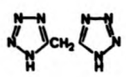
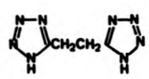
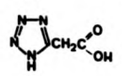
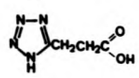
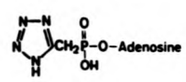
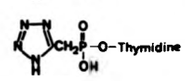
3.2.1 Effect of Heterocyclic Pyrophosphate Analogues on HSV-Induced DNA Polymerases

Table 3.2.1 shows the heterocyclic compounds studied and lists the ID_{50} values (μM) obtained against HSV-1 KOS strain DNA polymerase and HSV-2 3345 strain DNA polymerase. Compounds (1)-(4) and PAA were also tested against the DNA polymerase of the HFEM strain of HSV-1.

As shown by Table 3.2.1, most of the compounds are the nitrogen heterocyclic analogues of PAA or malonic acid, compounds (1),(4) and (3) showing a progressive decrease in their respective heterocyclic ring nitrogen acidities. Of these compounds the 1(H)-tetrazole analogue of PAA, 5-(phosphonomethyl)-1(H)-tetrazole (1) was most active against HSV-1 and HSV-2 DNA polymerases although at considerably higher concentrations than required for PAA. The corresponding less acidic triazole analogue (4) exhibited some activity against both enzymes (see Fig. 3.2.1) whereas blocking the acidic nitrogen function as in (3) virtually abolishes antiviral activity. The tetrazole analogue of 3-phosphonopropionic acid (2) was inactive, as were the bistetrazoles and tetrazole carboxylic acids (5)-(8).

The nucleoside-5' esters of (1) show weak activity in tissue culture but no significant inhibition

Table 3.2.1 Structures of heterocyclic pyrophosphate analogues and ID_{50} values obtained.

Compound		DNA pol ID_{50} μM				plaque redn. ID_{50} mg/ml , mM		
		HSV-1 KOS	HSV-1 HFEM	HSV-2 3345	∝ (mM)	HSV-1 KOS	HSV-2 3345	pK_d' Zn^{2+}
PAA		10	8	10	0·04	0·022 , 0·157	0·025 , 0·178	5·5
1		220	180	275	>1	0·40 , 1·72	0·35 , 1·50	5·6
2		>1000	>1000	>1000	>1	>1	>1	4·6
3		870	800	>1000	>1	>1	>1	-4
4		380	370	520	>1	0·7 , 3·01	0·82 , 3·53	4·7
5		>1000	–	>1000	>1	>1	>1	<4
6		>1000	–	>1000	>1	>1	>1	<4
7		>1000	–	>1000	>1	>1	>1	<4
8		>1000	–	>1000	>1	>1	>1	<4
9		>1000	–	>1000	>1	0·85 , 2·06	0·9 , 2·18	–
10		850	–	900	>1	0·7 , 1·81	0·72 , 1·87	–

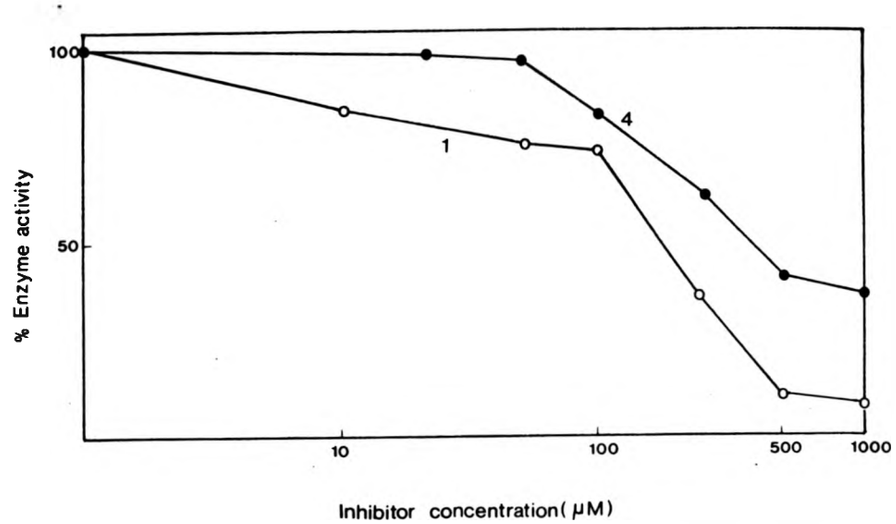


Fig. 3.2.1 A typical dose response curve showing the difference in the activities of 5-(phosphonomethyl)-1(H)-tetrazole (o) and 3-(phosphonomethyl)-1,2,4-triazole (●).

of the DNA polymerases is exhibited.

Also shown in Table 3.2.1 are the pyrophosphate analogue - zinc ion stability constants which were determined. The most effective zinc chelating compound of the phosphonates was (1) which had a pK_d, value of 5.6 (PAA had a pK_d, of 5.5 under the same conditions).

3.2.2 Effect of Compounds on HSV Plaque Formation

Of the heterocyclic phosphonates only 5-(phosphonomethyl)-1(H)-tetrazole (1) and 3-(phosphonomethyl)-1,2,4-triazole (4) had any effect on HSV-1 or HSV-2 plaque formation, (1) being the most effective with an ID_{50} of 0.35 mg/ml (1.5 mM) against HSV-2.

The nucleoside-5' esters exhibit weak activity against HSV-1 and HSV-2 plaque formation, the most effective, thymidine-5'-(5-phosphonomethyl)-1(H)-tetrazole (10) inhibited HSV-1 plaque formation by 50% at 0.7 mg/ml (1.81 mM).

3.2.3 Effect of HSV-1 Infection on the Cellular Uptake of Pyrophosphate Analogues

As shown by Fig. 3.2.3 A and Fig. 3.2.3 B, infection of Vero cells with HSV-1 18 CI (PAA^r) results in an increase in the uptake of both PAA and 5-(phosphonomethyl)-1(H)-tetrazole (1) relative to uninfected cells. The level of uptake, as indicated by label recovered in solubilised cells, reaches a maximum 6-7 hours after infection, when up to 1.5×10^6 nmoles/10^6 cells was recovered.

A direct comparison of the two compounds studied (Fig. 3.2.3 C) indicates similar levels of uptake into infected cells, a slight increase being observed for 5-(phosphonomethyl)-1(H)-tetrazole over PAA in the same conditions.

Fig. 3.2.3 Effect of HSV-1 (18CI) infection on the cellular uptake of 5-(phosphonomethyl)-1(H)-tetrazole (A) and PAA (B) [Δ-uninfected cells, o-infected cells].
A comparison of the uptake of PAA (Δ) and 5-(phosphonomethyl)-1(H)-tetrazole (o) into HSV-1 (18CI) infected cells (C).

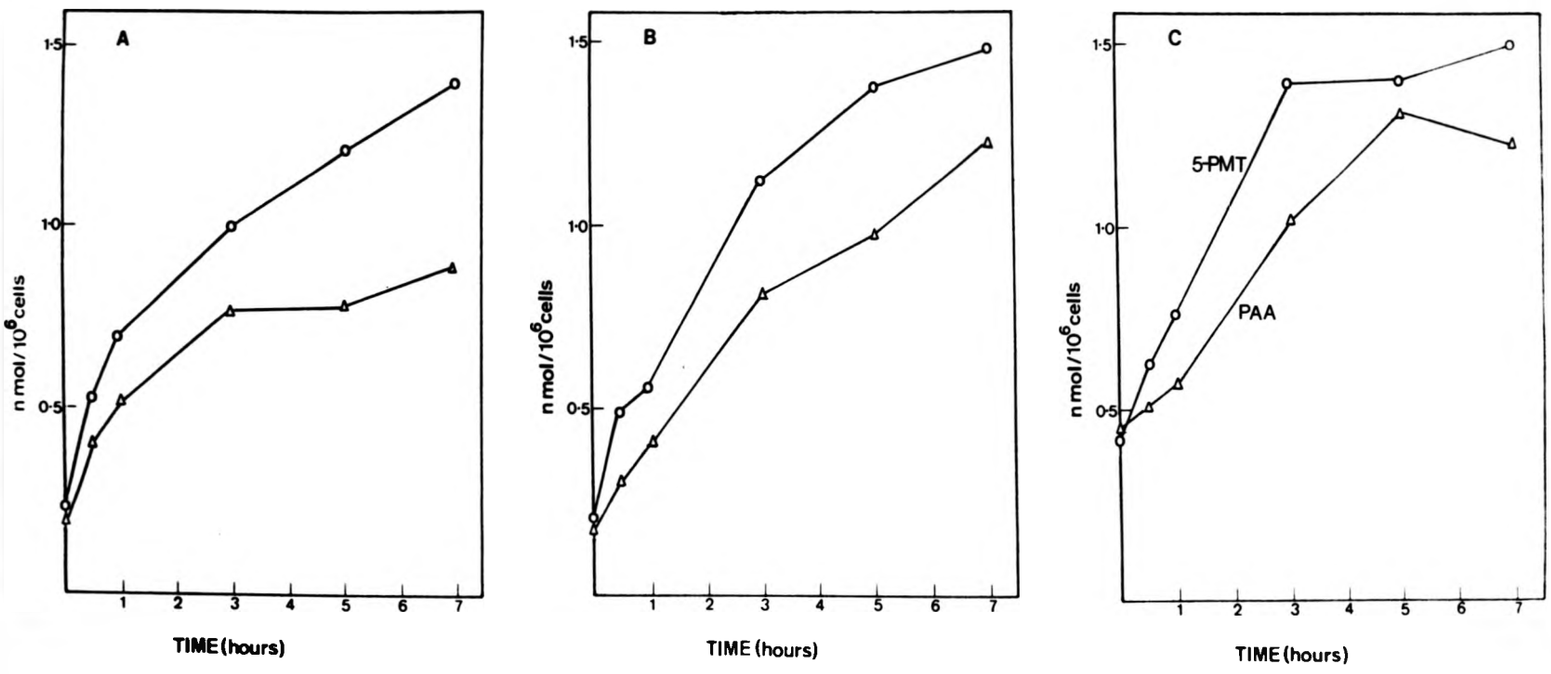


Fig. 3.2.3

3.3 DISCUSSION

Contrary to a previous report (Yaouanc, *et al.*, 1980), 5-(phosphonomethyl)-1(H)-tetrazole (1) was found to be inferior to PAA as an inhibitor of both HSV-1 and HSV-2 induced DNA polymerases and of HSV-1 and HSV-2 plaque formation. The rigid structural requirements for activity of pyrophosphate analogues against HSV-induced DNA polymerases previously reported (Eriksson, *et al.*, 1980; Mao, *et al.*, 1985) are thus also confirmed for the series of heterocyclic pyrophosphate analogues studied. The close similarities in the properties of tetrazoles and carboxylic acids, their similar spatial requirements, and moreover the very similar zinc chelating abilities of PAA (pK_d, = 5.5) and 5-(phosphonomethyl)-1(H)-tetrazole (pK_d, = 5.6), are therefore insufficient to result in similar levels of antiherpes activity.

The heterocyclic phosphonate-zinc ion stability constants determined show that only the compounds which form reasonably stable complexes (pK_d, = 4.5-6) are active against the herpesvirus-induced DNA polymerases. Replacement of the phosphonate function by the tetrazole moiety or carboxylic acid group results in a dramatic decrease in zinc binding and these compounds are devoid of antiviral activity. These results suggest that these compounds could act against the HSV-induced enzyme by virtue of coordination with an essential metal ion

(probably zinc) in the polymerase, which has not been established in previous studies in which good zinc chelating compounds have only been active against influenza RNA transcriptase activity.

The presence of an acidic heterocyclic ring nitrogen atom is important for activity. Replacement of the tetrazole ring in 5-(phosphonomethyl)-1(H)-tetrazole by the similar but less acidic triazole ring in 3-(phosphonomethyl)-1,2,4-triazole (4) or alkylation of the tetrazole ring in 2-ethyl-5-(phosphonomethyl)-tetrazole (3) results in a progressive decrease in their respective activities. Both compounds (3) and (4) also show a decrease in pK_d, value, however, both are still able to complex with zinc ions, perhaps by coordination through the available non-acidic nitrogen atoms to form six-membered chelate rings (Fig. 3.3).

Fig. 3.3 Possible zinc chelates of 3-(phosphonomethyl)-1,2,4-triazole (4) and 2-ethyl-5-(phosphonomethyl)-tetrazole (3).

5-(Phosphonoethyl)-1(H)-tetrazole (2) is able to form zinc complexes and has a similar pK_d, to (4) but is less active against the HSV-induced DNA polymerases. A decrease in antiviral activity on increasing the distance between the tetrazole and phosphono groups is in agreement with previous reports that elongation of the carbon chain between carboxylic acid and phosphono groups results in loss of activity (Leinbach, *et al.*, 1976; Eriksson, *et al.*, 1980).

None of the compounds studied had any effect on calf thymus DNA polymerase α, the active compounds therefore retained specific antiviral activity despite higher concentrations being required than for PAA to exhibit antiherpesvirus activity.

The highly charged nature of the phosphonates studied probably accounts for the much higher concentrations required for antiviral activity in tissue culture, it was therefore of interest to synthesise compounds which might show an improved uptake into cells and therefore higher antiviral activity in cells or *in vivo*.

With this in mind, and considering the reported antiviral activity of nucleoside esters of PAA (Heimer and Nussbaum, 1977), the adenosine-5' and thymidine-5' esters of (1) were prepared. These compounds were inactive as inhibitors of HSV-induced DNA polymerase but did show weak activity in tissue culture tests at comparable concentrations to (1) in the same assay system. These results suggest that

the nucleoside-5' esters could inhibit virus replication via a mechanism other than DNA polymerase inactivation. Alternatively, hydrolysis by enzymes inside infected cells to give the free phosphonate may result in the observed activity in cell culture systems. The esters are stable in the DNA polymerase assay conditions as demonstrated by t.l.c., however no studies on their stability to cellular enzymes and conditions have been undertaken.

Since synergism of mixtures of nucleoside antiviral agents and pyrophosphate analogues is known (Smith, *et al.*, 1982), compounds in which the two types of molecule are linked by a chemical bond as in nucleoside-5' esters, in which the nucleoside itself is an antiviral agent, for example 5'-esters of BVDU, may prove effective, particularly with regard to combating resistance. Nucleoside-5' esters of PFA have recently been investigated as possible reverse transcriptase inhibitors which might show greater specificity than PFA or PAA (Robins, 1984).

An investigation into the uptake of PAA and 5-(phosphonomethyl)-1(H)-tetrazole into infected and uninfected cells was undertaken in order to examine any differences in transport across cellular membranes between the tetrazole analogue and PAA and at the same time to determine whether any differences in the cellular uptake of phosphonates occur after viral infection. It was found that modification of

membrane permeability to both PAA and (1) appears to occur during infection with HSV-1, reaching a maximum 6 to 7 hours after infection.

The initial problem in this study was the choice of HSV strain. Initial experiments on HSV-1 (KOS) infected cells showed little difference in PAA uptake compared to uninfected cells. However, the concentration of PAA required to give sufficient radioactivity in cells to be accurately counted was at antiviral levels and therefore the possibility of virus inhibition and therefore inhibition of any virally-induced cell-permeabilisation could not be ruled out. The use of a PAA resistant strain, HSV-1 18CI, enables the use of higher dosage and therefore higher counts of [2-^{3}H]-PAA and (1) and allows the measurement of membrane permeability changes to pyrophosphate analogues during the normal course of infection.

Virally mediated changes in membrane permeability to small molecules is a widespread phenomenon (Carrasco and Smith, 1980) and has been reported for a number of viruses including paramyxoviruses (Benedetto, *et al.*, 1980) and herpesviruses (Fritz and Nahmias, 1967; Benedetto, *et al.*, 1980), also picornavirus, rhabdoviruses and togaviruses, taking place early as well as late in infection.

Some "impermeant" inhibitors of protein synthesis such as the GTP analogue guanylyl (β,γ-

methylene-)-diphosphonate ($GppCH_2p$) are said to inhibit protein synthesis in virally-infected cells but not uninfected cells due to an increased permeability across their plasma membrane (Carrasco, 1978; Carrasco and Lacal, 1983). This has, however, been disputed in another report (Gray and Pasternak, 1984) in which the uptake of sugars and amino acids as well as [^{3}H]$GppCH_2p$ was reported to be unaffected by infection with a variety of viruses.

In this study the uptake of [2-^{3}H]PAA and (1) increased soon after infection and reached a maximum (20% higher) late in infection. It appears, therefore, that some sort of modification of the plasma membrane does occur after infection. No conclusions can be drawn as to whether there occurs an effect on an uptake mechanism or merely an increase in the permeability of cells. Generalised membrane leakage would result in a gradual redistribution of ions and small molecules in the cells and the medium, with certain molecules being retained in the cell due to interactions with cellular or viral components, which would include the DNA polymerase. Both the initial and final steps in viral infection involve interactions with the cell surface. If the initial adsorption and entry of the virus alters the membrane permanently, this would account for an immediate difference in the uptake of small molecules which would proceed until the onset of cytopathic effect, after which cells leak in

general manner before lysis.

The bulk of herpesvirus DNA is made 3 to 6 hours post-infection (Roizman, 1969) and therefore the observed increase in permeability seems to occur too late to be exploited by DNA polymerase inhibitors.

Both PAA and (1) are taken up to similar extents in HSV-1 infected cells, (1) being taken up slightly more readily than PAA, thus confirming that the difference in the activities of these two compounds is a consequence of differences in HSV-induced DNA polymerase inhibition alone, rather than due to differences in their cellular uptake. Indeed, the uptake of (1) compares favourably with PAA, possibly due to the tetrazole group's similarity to some nucleoside bases or perhaps histidine, for which there may be other transport mechanisms.

CHAPTER 4

INFLUENZA VIRUSES

4.1 METHODS

4.1.1 Virus Stocks

Influenza A/X49 (H3N2), recombinant; [A/England/864/75 X A/PR/8/34] was a gift from Dr. N. J. Dimmock, *et al.*, Department of Biological Sciences, University of Warwick, Coventry, U.K. Influenza A/X31 (H3N2), recombinant; [A/PR/8/34 (H1N1) X A/Hong Kong/1/68 (H3N2)], influenza A/RI5 (H2N2) [A/RI/5^{+}/57] and influenza A/WS (H1N2) [A/WS/33] were gifts from Dr. K. Katrak, Roche Products Limited, Welwyn Garden City, U.K.

Abbreviations: PR = Puerto Rico, WS = Wilson Smith.

4.1.2 Preparation of RNA Transcriptase Assay Virus Stocks

The influenza A/X49 strain was used for the preparation of the enzyme assay stocks and was grown in the allantoic sacs of fertile hen's eggs essentially as described by Kelly and Dimmock (1974). Eleven-day old embryonated hen's eggs were inoculated with infected allantoic fluid (0.1 ml of a 10^{-3} dilution in PBS). The eggs were incubated (33°C/48 hours) and then chilled (-20°C/2 hours). The allantoic fluid was

collected and centrifuged (3000 r.p.m./20 minutes) to remove unwanted egg membranes; from this point onwards all procedures were carried out at 0-4°C. The supernatant was removed and the virus was pelleted by centrifugation (21 000 r.p.m./90 minutes, 6 x 300 ml rotor). The supernatant was discarded and the virus pellet allowed to soak overnight in PBS. The virus pellet was then resuspended in PBS (10 ml) and centrifuged (2500 r.p.m./10 minutes) before application onto a velocity gradient of 15-45% (w/v) sucrose in buffer (60 ml, 10 mM *Tris*-HCl, 150 mM NaCl, pH 7.4) and centrifuged (22 000 r.p.m./1 hour, 3 x 65 ml swing-out rotor). The diffuse virus band was collected by bottom puncture and the sucrose diluted with PBS and layered onto an equilibrium gradient of 30-70% (w/v) sucrose in buffer (60 ml, 10 mM *Tris*-HCl, 150 mM NaCl, pH 7.4) and centrifuged (20 000 r.p.m./overnight, 3 x 65 ml swing-out rotor). The virus band was collected, diluted with PBS and the virus pelleted by centrifugation (30 000 r.p.m./2 hours, 8 x 50 ml rotor). The supernatant was discarded and the pellet allowed to soak overnight in PBS before resuspending the virus in buffer (3 ml, 400 µM *Tris*-HCl, pH 8.0) and frozen as aliquots at -70°C and thawed once prior to use.

4.1.3 Influenza RNA Transcriptase Assay

RNA dependent RNA polymerase (transcriptase) activity was assayed in a 200 µl reaction mixture

containing 50 mM *Tris*-HCl, pH 8.0, 5 mM $Mg(CH_3CO_2)_2$, 150 mM KCl, 5 mM dithiothreitol, 0.4 mM ApG, 0.25% (v/v) Nonidet P-40, 0.4 mM each of ATP, CTP, GTP and [^{3}H]-UTP (5 µCi) and purified virus (10 µl). Mixtures were kept at 4°C until addition of virus, which was followed by incubation for 1 hour at 30°C. Reactions were stopped by the addition of 200 µl of saturated sodium pyrophosphate solution followed by cold TCA (2 ml, 10% w/v) and the mixtures kept on ice for 2 hours after thorough agitation. Precipitated material was collected on Whatman GF/C discs which were wetted with cold 10% (w/v) TCA, washed with 2 x 10 ml cold 10% (w/v) TCA, 1 x 10 ml ethanol, 1 x 5 ml ether and oven dried before counting in a toluene-based scintillant. Semi-logarithmic dose-response curves were drawn for each compound and ID_{50} values determined.

4.1.4 Plaque Reduction Assay

Madin-Darby canine kidney (MDCK) cells were seeded onto 5 cm plastic culture dishes (in duplicate) at 3 x 10^6 cells/plate. Cells were grown at 37°C in a humidified atmosphere of 5% CO_2 in air until confluent in maintenance medium (DMEM supplemented with 10% NCS, 4 mM L-glutamine, and penicillin/streptomycin (50 units/ml). The confluent monolayers were washed with PBS and aspirated to dryness. Virus suspension (100 µl, 60 p.f.u.) was added to the cell sheets and

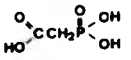
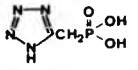
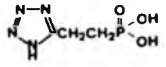
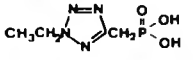
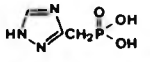
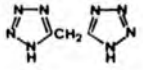
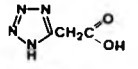
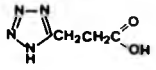
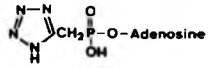
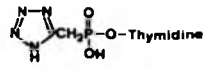
Compound		Influenza RNA pol ID_{50} (μM)	pK_d Zn^{2+}	pK_d Mg^{2+}	Plaque reduction(%) at 500 μM
PAA		350	5·5	4·8	8
1		275	5·6	4·4	25
2		550	4·6	<4	0
3		>1000	~4	<4	–
4		580	4·7	–	5
5		>1000	<4	–	–
6		>1000	<4	–	–
7		>1000	<4	–	–
8		>1000	<4	–	–
9		>1000	–	–	30
10		>1000	–	–	–

Table 4.2.1 Structures of heterocyclic compounds and ID_{50}, pK_d, values obtained.

left for 15 minutes at room temperature, followed by the addition of drug dilution in PBS (300 µl) and the cell sheets left for a further 45 minutes at room temperature. Cells were then overlaid with an agar medium (3.6 ml) containing 0.1% BSA, 2.5 µg/ml crystalline trypsin and 0.1% DEAE Dextran. The plates were incubated at 33°C for 4-6 days, stained with neutral red and the plaques counted. The percentage of plaque inhibition relative to the infected control (no drug) plates was determined for each drug concentration and the 50% inhibitory concentration (ID_{50}) values were calculated from dose response curves.

4.2 EXPERIMENTAL AND RESULTS

4.2.1 Effect of Heterocyclic Pyrophosphate Analogues on Influenza RNA Transcriptase Activity

Table 4.2.1 shows the ID_{50} values of compounds tested and their zinc ion stability constants. Of the heterocyclic phosphonates tested, 5-(phosphonomethyl)-1(H)-tetrazole (1) was the most active with an ID_{50} of 275 µM, under the same assay conditions PAA has an ID_{50} of 350 µM. 5-(Phosphonoethyl)-1(H)-tetrazole (2) and 3-(phosphonomethyl)-1,2,4-triazole (4) were weaker inhibitors of the transcriptase.

The nucleoside-5' esters of (1) were inactive as inhibitors of influenza RNA transcriptase, as were all compounds with low zinc ion stability constants

(pK_d, ≤ 4).

4.2.2 Effect of Heterocyclic Phosphonates on Influenza A/X49 Replication

Compounds (1), (2), (4), (9) and PAA were evaluated for antiviral activity in MDCK cells by a simple plaque reduction assay, the results of which are presented in Table 4.2.2(i).

Compound	% Plaque Number		
	1000 μM	500 μM	250 μM
PAA	67	92	100
(1)	55	75	84
(2)	100	100	100
(4)	89	95	100
(9)	57	70	81

Table 4.2.2(i) Plaque reduction by heterocyclic phosphonates against influenza A/X49 in MDCK cells

Compounds which were active were so at concentrations at least five times greater than required for inhibition of the RNA transcriptase, again reflecting the poor uptake of these compounds, also observed in studies with herpesviruses in Vero cells (Chapter 3). Adenosine-5'-(5-phosphonomethyl)-1(H)-tetrazole (9) however, was more active in tissue culture than against the transcriptase enzyme.

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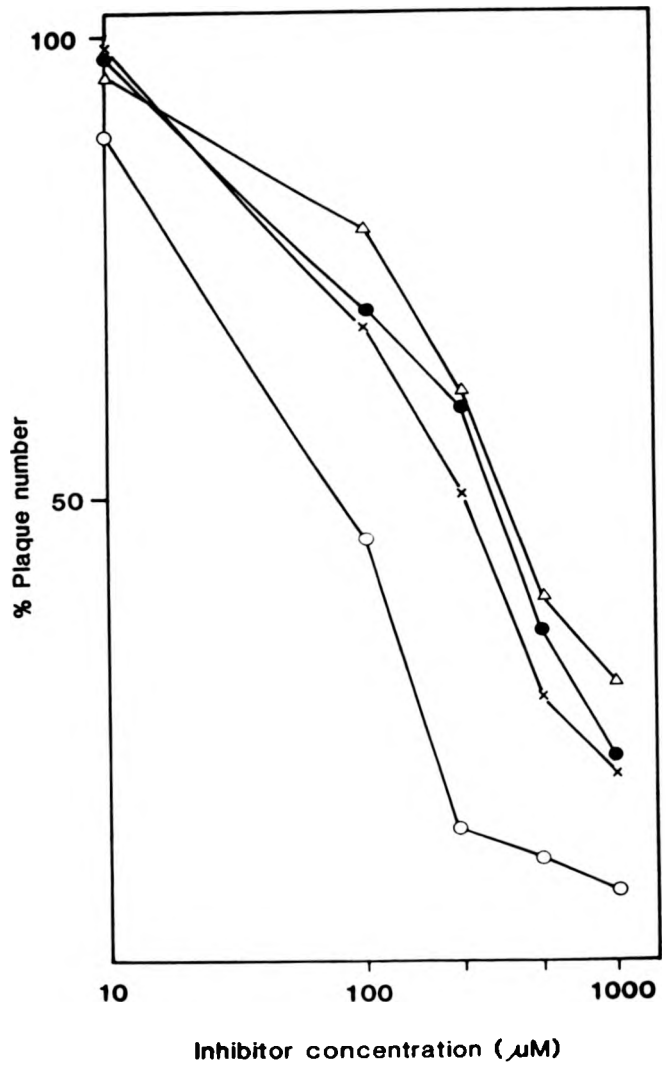


Fig. 4.2.3(i) Inhibition of plaque formation by strains of influenza virus A in MDCK cells by PFA, A/X49 (Δ), A/X31 (X), A/R15 (●), A/WS (o).

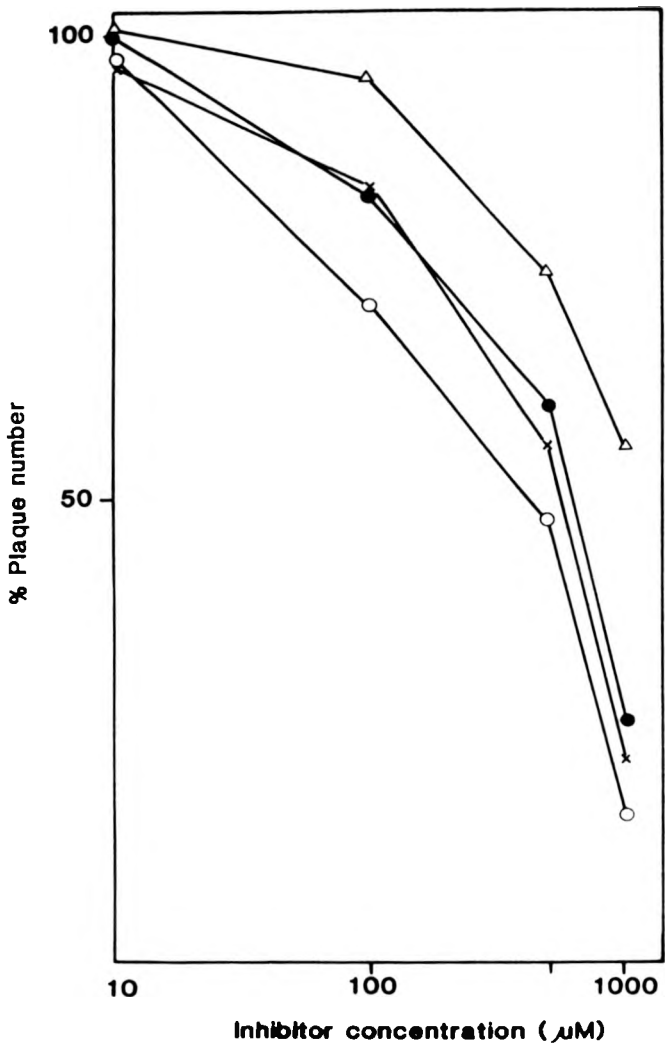


Fig. 4.2.3(ii) Inhibition of plaque formation by strains of influenza virus A by 5-(phosphonomethyl)-1(H)-tetrazole, A/X49 (Δ), A/X31 (X), A/R15 (●), A/WS (o).

The toxicity of these compounds to MDCK cells was tested at concentrations up to 8 mM for 3 days and any observable cytotoxicity noted. Results are presented in Table 4.2.2(ii).

Compound	Concentration (μM)				
	8000 μM	4000 μM	2000 μM	500 μM	250 μM
PAA	+	(+)	-	-	-
(1)	(+)	-	-	-	-
(2)	(+)	-	-	-	-
(4)	-	-	-	-	-
(9)	-	-	-	-	-
PFA	+	(+)	-	-	-

Table 4.2.2(ii) Cellular toxicity of pyrophosphate analogues, - no observable cytotoxitity, (+) slight cell damage observable, + cellular cytotoxicity apparent.

4.2.3 Effect of PFA and 5-(Phosphonomethyl)-1(H)-tetrazole (1) on the Replication of Various Strains of Influenza A Virus in MDCK Cells by Plaque Reduction Assay

Figures 4.2.3(i) and 4.2.3(ii) show the dose response curves obtained when PFA and (1) were evaluated for antiviral activity against a number of different strains of influenza A virus in MDCK cells. The ID_{50} values obtained are presented below (Table 4.2.3).

Influenza Virus Strain	ID_{50} (μM)	
	PFA	(1)
A/X49	380	> 1000
A/X31	340	440
A/RI5	270	600
A/WS	85	560

Table 4.2.3 Inhibition of influenza A strains in MDCK cells by PFA and (1).

4.3 DISCUSSION

The assay system used in this study utilised detergent-disrupted virus preparations. Virus treated in this manner makes available an RNA transcriptase activity which can be assayed in the presence of adenylyl-(3'-5')-guanosine (ApG) as primer and using endogenous RNA as template. The reactions carried out by this transcriptase complex, containing PA, PB_1, PB_2 and other components, has not yet been fully characterised and the enzyme kinetics found to be complex (Cload, 1983). For the purposes of this study, ID_{50} values are regarded as concentrations required to inhibit the whole transcriptase complex by 50% and cannot be related to specific stages in the polymerisation reaction.

5-(Phosphonomethyl)-1(H)-tetrazole (1) was the most effective of the compounds studied, inhibiting influenza A/X49 RNA transcriptase activity by 50%

at a concentration of 275 μM. Under the same conditions PAA inhibited the RNA transcriptase by 50% at 350 μM. The increased activity of (1) against this enzyme appears to be related to its zinc ion binding ability, a small increase in pK_d, being exhibited by (1) (pK_d, = 5.6) over PAA (pK_d, = 5.5). Furthermore, the observed ID_{50} values for compounds (1)-(8) are close to those that could be predicted from Fig. 1.3.6.3(i), again implicating zinc as having an essential role in these enzymes.

Although magnesium ions are essential for the RNA transcriptase activity, these compounds do not exert their inhibition by complexing with magnesium ions and removing them from solution, since the concentration of these ions in the polymerisation reactions is 5 mM, while compounds are active at concentrations at least ten-fold lower.

The magnesium binding properties of some of the active compounds were studied, the pK_d, (Mg^{2+}) values being obtained by the same gel-filtration and atomic absorption method as used for pK_d, (Zn^{2+}) determinations. The compounds were indeed found to be poor chelators of magnesium ions compared to zinc ions. It is interesting to note that while (1) forms slightly stronger zinc complexes than PAA, its magnesium binding ability seems to be significantly lower. This is most likely to be due to the replacement of the oxygen ligand, a "hard" base

(Pearson, 1968), in PAA by the "softer" heterocyclic nitrogen ligand of (1), reducing the tendency of (1) to form complexes with "hard" metal ions such as magnesium (this is discussed further in Section 5.5).

5-(Phosphonomethyl)-1(H)-tetrazole (1) was a more effective inhibitor of influenza A/X49 RNA transcriptase than PAA but a much less effective inhibitor of calf thymus DNA polymerase α (ID_{50} = > 1000 μM) and therefore shows much greater selectivity in its enzyme inhibition, indeed PAA inhibits the RNA transcriptase at higher concentrations than required to inhibit DNA polymerase α; a property not exhibited by any of the heterocyclic compounds studied.

Little inhibition of influenza A/X49 replication in tissue culture plaque reduction tests was observed with most compounds, 5-(phosphonomethyl)-1(H)-tetrazole (1) was however, more active than PAA, inhibiting plaque formation by 25% at a concentration of 500 μM. The only other compound with comparable activity was the adenosine-5' ester (9) which inhibited plaque formation by 30% at 500 μM despite being inactive as an inhibitor of the viral RNA transcriptase. Nucleoside-5' esters of (1) showed similar patterns of inhibition in the herpesvirus assays, and is presumed to be due to hydrolysis to give free phosphonates in tissue culture systems.

None of the heterocyclic pyrophosphate analogues studied were toxic to MDCK cells after

3-4 days at concentrations up to 4 mM, four times greater than the highest concentration of test compound used in the plaque reduction assays. Both PAA and PFA were more toxic than the heterocyclic compounds at the higher concentrations, probably due to the greater susceptibility of cellular DNA polymerase α to these compounds and also the lower acidity of compounds (4) and (9).

Varying sensitivities of various strains of herpesviruses to pyrophosphate analogues has been noted in this study and in a number of other reports. For example, strains of HSV-1 and HSV-2 have been isolated which show complete resistance to PAA and PFA, and this resistance has been correlated with changes in the DNA polymerases induced by these viruses (Eriksson and Öberg, 1979; Hay and Subak-Sharpe, 1976; Honess and Watson, 1977). Strains which are not resistant to these compounds can also show variation in susceptibility to these compounds, and the DNA polymerases of several strains of HSV-1, when inhibited with PFA, show a range of ID_{50} values from 0.4-3.5 μM (Eriksson and Öberg, 1979; Helgstrand *et al.*, 1978).

The sensitivities of various strains of influenza virus A to different concentrations of PFA and (1) revealed that the A/WS strain was the most sensitive and A/X49 the least sensitive strain tested. Variation in ID_{50} values obtained was not great

amongst strains A/X31, A/RI5 and A/X49, however, the A/WS strain was significantly more sensitive to PFA in particular. The reason for the difference in sensitivity is not known, no studies have been reported on the differences in the sensitivities to pyrophosphate analogues of RNA transcriptase enzymes from influenza viruses, nor on influenza virus multiplication, although it has previously been observed that the effect of the antiviral drugs rimantadine and ribavirin in combination on influenza virus replication in cell culture is dependent on the strain of virus used (Hayden, *et al.*, 1980). Previous studies have also shown that there is considerable strain variability in the amantadine susceptibility of influenza viruses, and this has been reported to decrease with increasing passage level in animals (Tisdale and Bauer, 1975). In the present study the more contemporary strain of influenza A used, influenza A/RI5 was no more sensitive to the pyrophosphate analogues than the other strains.

Amantadine resistance is a stable genetic characteristic that relates to the gene coding for the M (matrix) protein (Lubeck, *et al.*, 1978), and variation in the three polymerase protein genes PA, PB_1, and PB_2, may potentially play an important role in susceptibility to pyrophosphate analogues, as well as in host range and virulence, although the extent of this has not yet been determined.

The differences observed in tissue culture

systems may well be due to factors other than sensitivities of the various RNA transcriptases to these compounds, for example, cellular penetration in cells infected with different strains may vary due to different viruses affecting cell membranes and cellular functions differently. Studies on isolated RNA transcriptase systems are obviously required to clarify the events leading to variation in susceptibility to pyrophosphate analogues.

CHAPTER 5

THIOPHOSPHONATES

5.1 SYNTHETIC CHEMISTRY

5.1.1 Sources of Chemicals

Monothiopyrophosphate (12) and bisthiopyrophosphate (13) were gifts from Dr. P. M. Cullis, Department of Chemistry, University of Leicester, U.K. Thio-analogues of PAA, PFA and methylenebisphosphonate were prepared as described in Section 5.2 from the corresponding trimethylsilyl esters which were gifts from Dr. S. Masson, University of Caen, France. Trimethylsilyl esters of thiophosphonates were synthesised as described in Appendix IV. *O*-Ethyl phosphonothioacetate (20) and ethyl phosphonodithioacetate (21) were gifts from Dr. S. Masson, and were prepared as described in Appendix V. The thio-analogue of 5-(phosphonomethyl)-1(H)-tetrazole (1) was synthesised as described in Section 5.2.

5.1.2 Synthetic Routes

Diethyl esters of thiophosphonates can be prepared by Michaelis-Becker reaction of the corresponding chloroalkyl intermediates with diethylthiophosphite (Scheme II, Fig. 5.1.2(i)). Diethylthiophosphite is synthesised by reaction of commercially

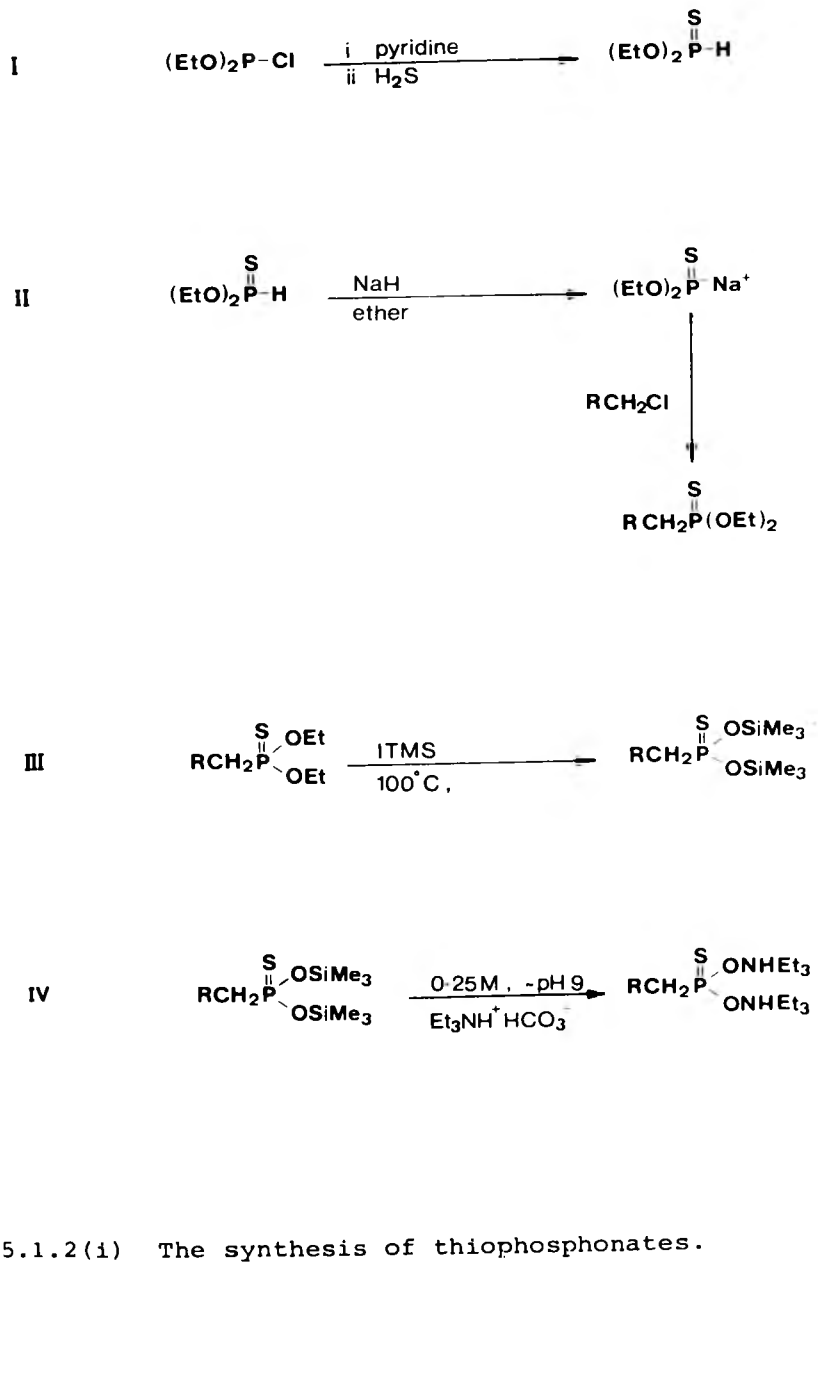


Fig. 5.1.2(i) The synthesis of thiophosphonates.

available diethylchlorophosphite (diethylphosphorochloridite) with gaseous hydrogen sulphide in benzene according to Scheme I (Fig. 5.1.2(i)). The final step in these synthetic routes involves dealkylation of diethyl thiophosphonates with ITMS, however more vigorous conditions are necessary for the dealkylation of thiophosphonates than required for dialkyl phosphonates, generally requiring temperatures of 100°C and longer reaction times (Scheme III, Fig. 5.1.2(i)). Removal of the extremely hydrolytically labile trimethylsilyl groups requires stirring the compound in aqueous triethylammonium bicarbonate (pH 8.7-9.5) in order to avoid partial desulphuration which can occur at lower pH and in the presence of iodine and water. The presence of bromine and water has indeed been used to induce the desulphuration of bisthiopyrophosphate (Cullis, 1983).

Triethylammonium salts of the thiophosphonates can then be purified by DEAE-Sephadex ion-exchange chromatography which satisfactorily separates the thiophosphonates from partially or fully desulphurised by-products.

5-(Thiophosphonomethyl)-1(H)-tetrazole (18) was thus prepared according to Fig. 5.1.2(ii) from the N-protected 5-(chloromethyl)-tetrazole also used in the synthesis of the tetrazole phosphonates as described in Section 2.

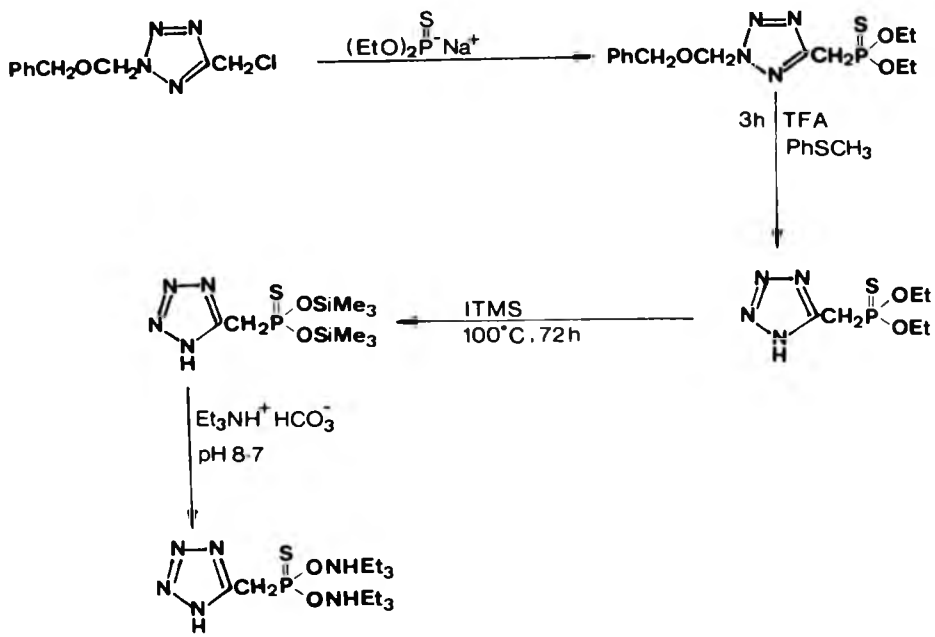


Fig. 5.1.2(ii) Route to 5-(thiophosphonomethyl)-1(H)-tetrazole (18).

Monothiopyrophosphate (12) and bisthiopyrophosphate (13) had been prepared as previously described (Cullis, 1983) as shown in Fig. 5.1.2(iii).

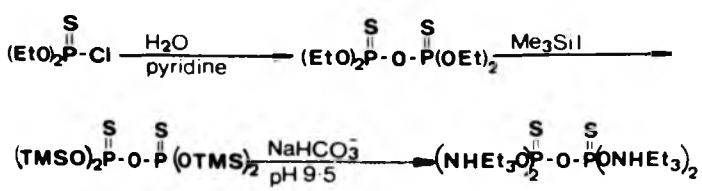


Fig. 5.1.2(iii) Synthesis of thiopyrophosphates.

5.2 EXPERIMENTAL

Diethylthiophosphite

Diethylthiophosphite was prepared according to the method of Krawiecki and Michalski (1960). A solution of diethylchlorophosphite (16 ml, 0.1 mol) and pyridine (8 ml, 0.1 mol) in benzene (75 ml) was stirred and hydrogen sulphide passed slowly through for 4 hours while the temperature was maintained at 5°C. Pyridinium hydrochloride was filtered off and the filtrate washed with 2 x 25 ml ice-cold water, dried and evaporated *in vacuo*. The residue was distilled and diethylthiophosphite obtained (12.1 g, 78%) b.p. 90-92°C/18 mm, ^{31}P n.m.r. ($CDCl_3$) δ 69.4 p.p.m.

5-(Thiophosphonomethyl)-1(H)-Tetrazole (18)

Sodium hydride (50% suspension in oil, 0.2 g, 4 mmol) was added to a solution of diethylthiophosphite (0.66 g, 4 mmol) in diethyl ether (20 ml) and the mixture heated under reflux for 30 minutes. The reaction was cooled to 0°C and 2-(benzyloxymethyl)-5-(chloromethyl)-tetrazole (1.2 g, 5 mmol) was added dropwise with stirring, which was continued for 48 hours. The ether was then removed *in vacuo* and the residue purified by silica gel chromatography (solvent A) to give diethyl 2-(benzyloxymethyl)-5-(thiophosphonomethyl)-tetrazole (1.3 g, 73%), ^{1}H n.m.r. ($CDCl_3$) δ 1.3 (6H, t, J = 6 Hz), 3.75 (2H, d, $^{2}J_{P-H}$ =

18 Hz), 4.15 (4H, m, J = 7 Hz), 4.6 (2H, s), 5.9 (2H, s), 7.3 p.p.m. (5H, m), ^{31}P n.m.r. ($CDCl_3$) δ 89 p.p.m., CIMS m/z 357 $[M + H]^+$.

Diethyl 2-(benzyloxymethyl)-5(thiophosphonomethyl)-tetrazole (500 mg, 1.4 mmol) was then stirred for 3 hours in a solution of trifluoroacetic acid (43 g, 380 mmol) and thioanisole (8.7 g, 70 mmol) at room temperature. Evaporation of excess TFA and thioanisole *in vacuo* afforded diethyl 5-(thiophosphonomethyl)-1(H)-tetrazole after purification by silica gel column chromatography (solvent C) (215 mg, 65%), ^1H n.m.r. ($CDCl_3$) δ 1.25 (6H, t, J = 6 Hz), 3.77 (2H, d, $^2J_{P-H}$ = 18 Hz), 4.1 p.p.m. (4H, m, J = 7 Hz), ^{31}P n.m.r. ($CDCl_3$) δ 86.8 p.p.m., CIMS m/z 237 $[M + H]^+$, 254 $[M + NH_4]^+$.

Diethyl 5-(thiophosphonomethyl-1(H)-tetrazole (150 mg, 0.6 m mol) was heated in a sealed tube at 100°C for 72 hours with iodotrimethylsilane (400 mg, 1.7 m mol). The silane was removed *in vacuo* and the residue treated with 2 x 10 ml of 0.25 M triethylammonium bicarbonate (pH 8.7), the sample being lyophilised between each treatment. The product was purified by ion-exchange chromatography on a DEAE-Sephadex A25 column (1.5 x 45 cm, Et_3NH^+ form) eluted with a gradient of 0.25-0.75 M triethylammonium bicarbonate (1.5 L, pH 8.7) to give 5-(thiophosphonomethyl)-1(H)-tetrazole as the bis(triethylammonium)salt (40 mg, 18%), ^1H n.m.r. (D_2O) δ 1.3 (18H, t, J = 6.5 Hz), 3.5 (2H, d, $^2J_{P-H}$ = 18 Hz),

4.15 p.p.m. (12H, q, J = 7 Hz), ^{31}P n.m.r. (D_2O) δ 59.9 p.p.m. analysis C, 38.52; H, 9.31; N, 21.5; $C_{14}H_{35}N_6O_2PS.H_2O$ requires C, 40.53; H, 9.31; N, 21.0.

Thiophosphonomethyl Phosphonate (15)

The silyl ester (3, Appendix IV, 0.5 g, 1 mmol) was stirred with 2 x 10 ml 0.25 M aqueous triethylammonium bicarbonate (pH 8.7), the sample being lyophilised between each treatment. The residue was purified by chromatography on a DEAE-Sephadex A25 ion-exchange column eluted with a gradient of 0-1.0 M triethylammonium bicarbonate to afford the triethylammonium salt of thiophosphonomethyl phosphonate (240 mg, 60%), which was then converted into the sodium salt using a Dowex 50 (H^+ form) ion-exchange column to give the sodium salt of thiophosphonomethyl phosphonate (130 mg, 55%), ^1H n.m.r. (D_2O) δ 2.6 p.p.m. (2H, dd, $^2J_{P-H}(O)$ = 20 Hz, $^2J_{P-H}(S)$ = 18 Hz), ^{31}P n.m.r. (D_2O) δ 57.55 (d), 18.15 p.p.m. (d), J_P = 8 Hz, analysis C, 4.96; H, 3.63; P, 25.9; $CH_4O_5P_2SNa_2\frac{1}{2}H_2O$ requires C, 4.90; H, 2.1; P, 25.28.

Methylene Bis-thiophosphonate (16)

Methylene bis-thiophosphonate was prepared as above from the corresponding silyl ester (4, Appendix IV) to give the bis(triethylammonium) salt

in 65% yield. ^{1}H n.m.r. (D_2O) δ 1.3 (18H, t, J = 6.5 Hz), 2.7 (2H, t, $^{2}J_{P-H}$ = 16 Hz), 4.15 p.p.m. (12H, q, J = 7 Hz), ^{31}P n.m.r. (D_2O) δ 60.3 p.p.m., analysis C, 37.1; H, 9.37; N, 6.71; P, 16.23; $C_{13}H_{36}N_2O_4P_2S_2\frac{1}{2}H_2O$ requires C, 37.2; H, 9.13; N, 6.68; P, 14.76.

Thiophosphonoacetic Acid (17)

Thiophosphonoacetic acid triethylammonium salt was prepared as described above from the silyl ester (6, Appendix IV) in 50% yield from the DEAE-Sephadex A25 column. ^{1}H n.m.r. (D_2O) δ 1.3 (9H, t, J = 6.5 Hz), 3.0 (2H, d, $^{2}J_{P-H}$ = 18 Hz), 4.2 p.p.m. (6H, q, J = 7 Hz), ^{31}P n.m.r. (D_2O) δ 57.8 p.p.m., analysis C, 37.73; H, 7.66; N, 5.64; $C_8H_{20}NO_4PS$ requires C, 37.34; H, 7.83; N, 5.44.

Thiophosphonoformic Acid (19)

The trimethylsilyl ester of thiophosphonoformic acid (500 mg, 1.4 mmol) was stirred in 5 ml of 0.5 M triethylammonium bicarbonate (pH 9.5) and after stirring for 15 minutes was immediately applied to a DEAE-Sephadex A25 column (Et_3NH^+ form, 1.5 x 50 cm) which was eluted with a gradient of 0.25-1.0 M triethylammonium bicarbonate (pH 9.5) to afford thiophosphonoformic acid as the triethylammonium salt (163 mg, 46%), ^{31}P n.m.r. (D_2O) δ 45.99 p.p.m., analysis C, 30.93; H, 8.28; N, 5.74; P, 12.43; $C_7H_{18}NO_4PS\frac{1}{2}H_2O$ requires C, 33.3; H, 7.59; N, 5.55; P, 12.3.

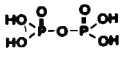
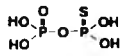
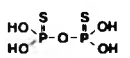
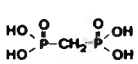
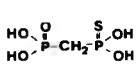
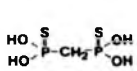
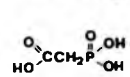
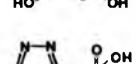
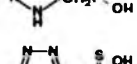
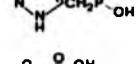
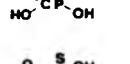
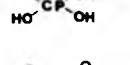
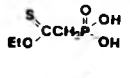
Compound		ID_{50} (μM)			pK_d Zn^{2+}
		Influenza RNA	HSV-1 DNA	Calf thymus DNA α	
11		125	>500	>500	5·7
12		60	>500	>500	5·4
13		33	>500	>500	>6
14		>500	>500	>500	5·3
15		>500	>500	>500	>6
16		350	>500	>500	>6
PAA		350	10	40	5·5
17		135	16	75	>6
1		275	220	>500	5·6
18		130	90	>500	>6
PFA		30	12	50	5·7
19		45	9	150	>6
20		>500	>500	n.d.	~5
21		400	>500	n.d.	5·5

Table 5.3 Structures of thiophosphonates and ID_{50} values obtained.

5.3 ANTIVIRAL ACTIVITIES OF THIOPHOSPHONATES EXPERIMENTAL AND RESULTS

5.3.1 Effect of Thio-analogues of Inorganic Pyrophosphate on Influenza A/X49 RNA Transcriptase Activity and HSV-1 (HFEM) DNA Polymerase Activity

Influenza A/X49 RNA transcriptase activity was assayed as described in Section 4.1.3. Table 5.3 shows the ID_{50} values determined for pyrophosphate (11, 125 μM), monothiopyrophosphate (12, 60 μM) and bisthiopyrophosphate (13, 33 μM), derived from the dose-response curves obtained (Fig. 5.3.1) against influenza A/X49 RNA transcriptase.

The compounds show a progressive (approximately two-fold) increase in antiviral activity upon the substitution of each phosphoryl bond with a thiophosphoryl bond, bisthiopyrophosphate (13) being four times more active than pyrophosphate (11) as an inhibitor of the RNA transcriptase.

HSV-1 (HFEM) DNA polymerase activity, assayed as described in Section 3.1.5, was unaffected by compounds (11), (12) and (13), (all ID_{50}'s > 500 μM).

5.3.2 Effect of Thio-analogues of Methylenebisphosphonate on Influenza A/X49 RNA Transcriptase Activity and HSV-1 (KOS) DNA Polymerase Activity

Methylenebisphosphonate (14) is devoid of antiherpes and anti-influenzal activity, however its thio-analogues show increasing activity against influenza RNA transcriptase and increasing zinc ion

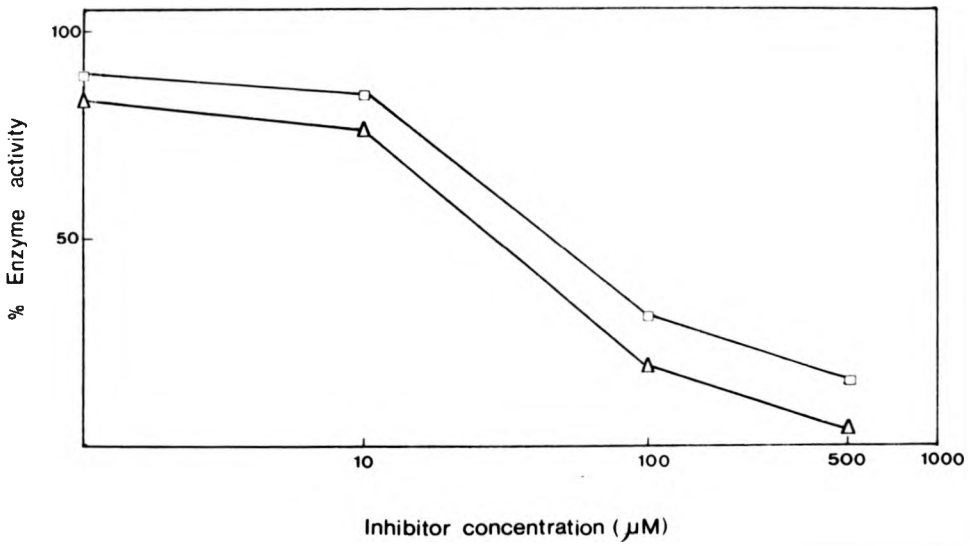


Fig. 5.3.1 Inhibition of RNA transcriptase of influenza virus (A/X49) by mono-(□) and bisthiopyrophosphate (Δ).

binding (ID_{50} (15) > 500 μM, pK_d, ~ 6; ID_{50} (16) = 350 μM, pK_d, > 6). No improvement in activity against HSV-1 (KOS) DNA polymerase activity was observed ((14), (15), (16) ID_{50}'s > 500 μM).

5.3.3 Effect of Thiophosphonoacetic Acid on Influenza A/X49 RNA Transcriptase Activity and HSV-1 (KOS) DNA Polymerase Activity

As shown in Table 5.3, replacement of a phosphoryl bond by a thiophosphoryl bond in PAA has a marked effect on the zinc ion stability constant obtained (PAA $pK_{d'}$ = 5.5, (17) $pK_{d'}$ > 6) and an improved activity against influenza RNA transcriptase activity. Thiophosphonoacetic acid has comparable antiherpes activity to PAA, inhibiting the HSV-1 (KOS) DNA polymerase by 50% at a concentration of 16 μM.

5.3.4 Effect of 5-(Thiophosphonomethyl)-1(H)-Tetrazole (18) on Influenza A/X49 RNA Transcriptase Activity and HSV-1 (KOS) DNA Polymerase Activity

5-(Thiophosphonomethyl)-1(H)-tetrazole (18) shows similar increases in activity and zinc-binding to those obtained for thiophosphonoacetic acid (17) over PAA, and inhibited influenza A/X49 RNA transcriptase activity by 50% at a concentration of 130 μM (Fig. 5.3.4). An increase in activity against HSV-1 (KOS) DNA polymerase was also observed, inhibition of this enzyme activity by 50% was caused by 5-(thiophosphonomethyl)-1(H)-tetrazole at a concentration of 90 μM.

5.3.5 Effect of Thiophosphonoformic Acid (19) on Influenza A/X49 RNA Transcriptase Activity and HSV-1 (KOS) DNA Polymerase Activity

Despite the increased zinc ion stability

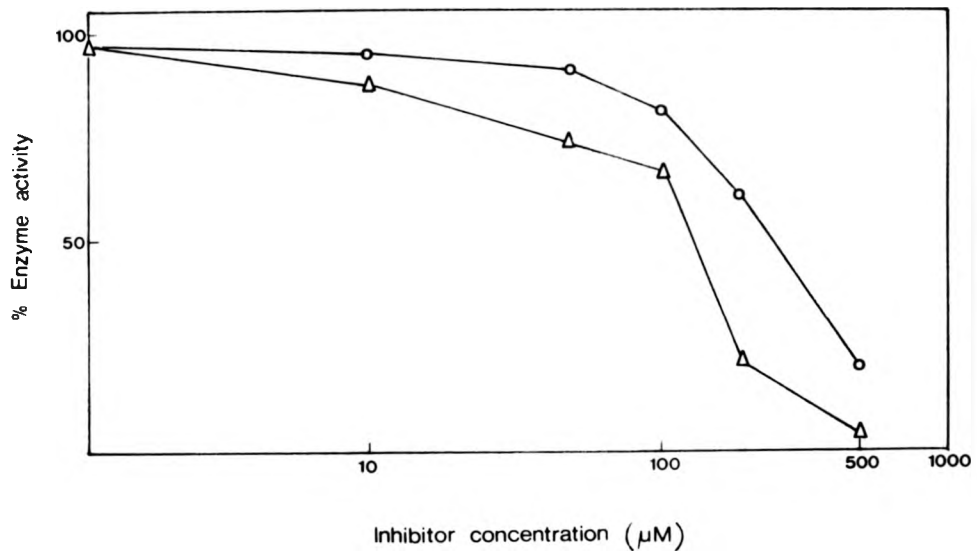


Fig. 5.3.4 Inhibition of RNA transcriptase of influenza A/X49 by 5-(phosphonomethyl)-1(H)-tetrazole (o) and 5-(thiophosphonomethyl)-1(H)-tetrazole (∆).

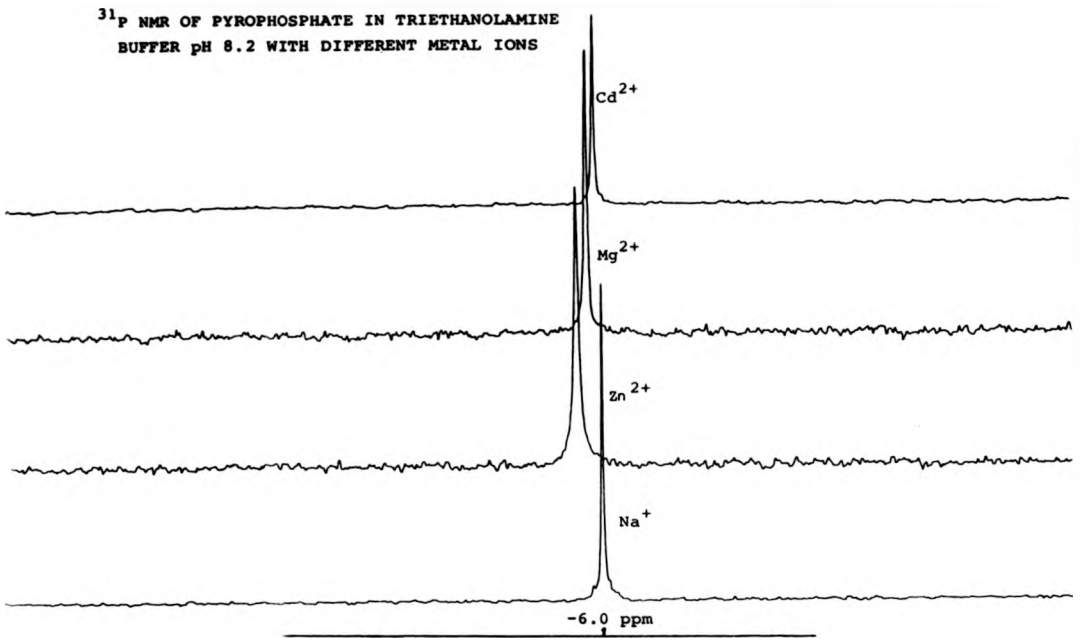
constant of (19) over PFA, anti-influenzal and antiherpes activity was little affected, a slight increase in activity against HSV-1 (KOS) DNA polymerase being observed (ID_{50} (19) = 9 μM, ID_{50} PFA = 12 μM).

5.3.6 Effect of Thioacetate Analogues of PAA on Influenza A/X49 RNA Transcriptase Activity and HSV-1 (KOS) DNA Polymerase Activity

Two PAA analogues - *O*-ethyl phosphonothioacetate (20) and ethyl phosphonodithioacetate (21) - were tested for antiviral activity and found to be weak inhibitors of the influenza RNA transcriptase activity, but were devoid of antiherpes activity.

5.4 ^{31}P N.M.R. MEASUREMENTS OF PYROPHOSPHATE AND BISTHIOPYROPHOSPHATE IN THE PRESENCE OF DIFFERENT METAL IONS

^{31}P n.m.r. measurements were carried out at 308 K and 36.44 MHz on a Bruker WH90 spectrometer, chemical shifts are recorded relative to H_3PO_4 (0 p.p.m.). Solutions of tetrasodium pyrophosphate or tetra-(triethylammonium)bisthiopyrophosphate (15 mg/ml) were prepared in 0.1 M triethanolamine hydrochloride buffer (pH 8.2), the latter being made up in water which had previously been passed down a Chelex 100 (1 x 10 cm) chelating column (Na^+ form, Biorad Inc.) to remove contaminating metal ions. To the pyrophosphate or bisthiopyrophosphate solution (1 ml, 1 equiv.) was added a solution in deuterium oxide (also treated with Chelex 100, 1 ml) which contained (0.8 equiv.) magnesium chloride, zinc chloride or cadmium bromide. The solutions were mixed, adjusted to pH 8.2 and the ^{31}P n.m.r. spectra were recorded (Fig. 5.4). Chemical


^{31}P NMR OF PYROPHOSPHATE IN TRIETHANOLAMINE
BUFFER pH 8.2 WITH DIFFERENT METAL IONS
Cd^{2+}
Mg^{2+}
Zn^{2+}
Na^{+}
-6.0 ppm

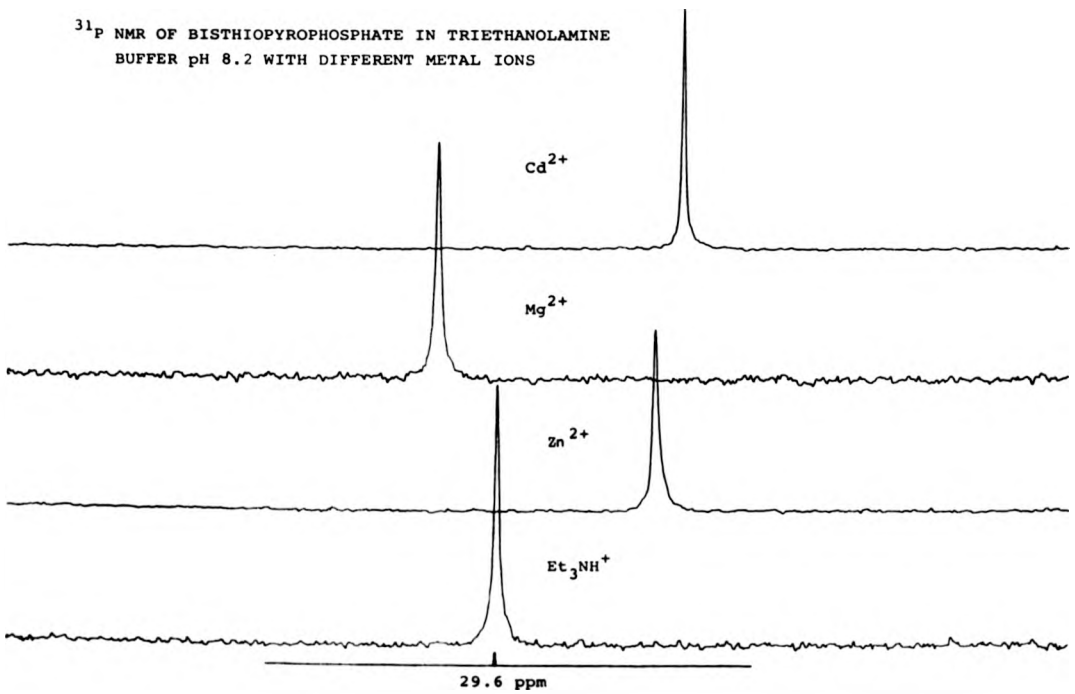

^{31}P NMR OF BISTHIOPYROPHOSPHATE IN TRIETHANOLAMINE
BUFFER pH 8.2 WITH DIFFERENT METAL IONS
Cd^{2+}
Mg^{2+}
Zn^{2+}
Et_3NH^{+}
29.6 ppm

Fig. 5.4

shifts are presented in Table 5.4.

Metal	P_2O_7	$P_2O_5S_2$
None	-6.045	29.693
Mg^{2+}	-5.254	32.418
Zn^{2+}	-4.839	21.849
Cd^{2+}	-5.643	20.714

Table 5.4 ^{31}P n.m.r. chemical shifts (p.p.m.) of solutions of pyrophosphate and bisthio-pyrophosphate at pH 8.2 after the addition of metal ions.

5.5 DISCUSSION

The apparent importance of zinc ion chelation in the antiviral activities of some pyrophosphate analogues prompted the investigation of compounds which might exhibit markedly different chelating properties without gross modifications in the structures of parent compounds. One way in which this can be achieved is by replacing the phosphoryl bond of phosphonates with the thiophosphoryl bond to give thiophosphonates. Replacement of an oxygen atom by the less electronegative and more polarisable sulphur atom has little effect on the molecular dimensions of the compound apart from the increased size of the sulphur atom and the PS bond being slightly lengthened and weaker than the PO bond (Saenger and Ekstein,

1970; Moritani, *et al.*, 1971). The thiophosphonates synthesised and tested, however, show marked increases in antiviral activity over the phosphono-analogues and also large increases in zinc ion stability constants are observed.

The most potent compound tested was bisthio-pyrophosphate (13), containing two thiophosphoryl bonds, which inhibited influenza A/X49 RNA transcriptase activity by 50% at a concentration of 33 μM. The series of pyrophosphate (11), monothiopyrophosphate (12) and bisthiopyrophosphate (13) show a progressive and approximately two-fold increase in anti-influenzal activity for each thiophosphoryl bond substituted. Bisthiopyrophosphate was thus four times more active than inorganic pyrophosphate against this enzyme and has the greatest zinc ion stability constant of the series. Similar patterns are observed for the series of methylenebisphosphonates (14)-(16). Although methylenebisphosphonate has no antiviral activity, replacement of the phosphoryl group with a thiophosphoryl group results in some inhibition of influenza A/X49 RNA transcriptase activity. Similar increases in antiviral activity and zinc binding are observed for thio-analogues of 5-(phosphonomethyl)-1(H)-tetrazole (1), PAA and PFA.

The fact that mono- and bisthiopyrophosphate show considerable antiviral activity in tissue culture against influenza A/X49 is also of importance (Hutchinson,

et al., 1985). Pyrophosphate is involved in many enzymic reactions in host cells and some pyrophosphate analogues such as dichloromethylene bisphosphonate are cytotoxic (Cload and Hutchinson, unpublished observations, 1983). It is therefore significant that the mono- and bisthiopyrophosphates do not appear to be cytotoxic to MDCK cells after 36 hours at concentrations at which they cause an appreciable reduction in the haemagglutinin titre of influenza virus A/X49. Mono- and bisthiopyrophosphate also cause significant inhibition of plaque formation at 50 μM.

The Principle of Hard and Soft Acids and Bases (HSAB, Pearson, 1968) can be used to explain the differences in metal ion chelating properties of the phosphonates and their thio-analogues. The so-called 'hard' bases (Table 5.5(i)) are donor atoms of high electronegativity, of low polarisability and hard to oxidise. 'Soft' bases are larger, of low electronegativity, high polarisability and easy to oxidise.

Hard	Soft
H_2O, OH^-, F^-, Cl^-	CN^-, RNC, CO
through $O^-(P(O)-O^-$,	through S (RS^-, R_2S, RSH)
RO^-, $RCOO^-$)	R_3P, R_3As, $(RO)_3P$,
ROH, R_2O	I^-, SCN^-, $S_2O_3^{2-}$
NH_3, RNH_2, N_2H_4	
Intermediate	
Amines binding through N (ranging from primary to tertiary)	

Table 5.5(i) Hard and soft bases (ligands) relevant to biological systems.

The softness of a ligand can therefore be regarded as describing the looseness with which they hold their valence electrons. The classification of Lewis acids (Table 5.5(ii)) shows that the 'hard' acids are acceptor atoms of small size, high positive charge and lacking unshared electron pairs in their valence shell (not all these properties need to be possessed by any one acid). These properties lead to high electronegativity and low polarisability.

Hard	Soft
H^+, Li^+, Na^+, K^+	Cu^+, Ag^+, Hg^+
Mg^{2+}, Ca^{2+}, Mn^{2+}	Pd^{2+}, Cd^{2+}, Pt^{2+},
Al^{3+}, Fe^{3+}, As^{3+}	
Intermediate	
Fe^{2+}, Co^{2+}, Cu^{2+}, Zn^{2+}	

Table 5.5(ii) Hard and soft acids (metal ions) relevant to biological systems

'Soft' Lewis acids are generally larger acceptor atoms of low positive charge and containing unshared pairs of p or d electrons in their valence shell. The Pearson rule is that 'hard' acids (in this case metal ions) bind to hard bases (ligands) and 'soft' acids prefer to bind 'soft' bases.

On the Pearson 'hard and soft' acid and base scale, zinc ions can be classified as 'intermediate-soft' and hence should form complexes with both hard

and soft ligands. Indeed, as shown by the complexing characteristics and *in vivo* roles of some essential metal ions (Table 5.5(iii)) nitrogen and sulphur ligands are preferred to oxygen as donor atoms (Ainscough and Brodie, 1976).

The thiopyrophosphates (12) and (13) contain a potential soft ligand (sulphur) and this can explain why these compounds are good chelators of zinc ions and therefore good inhibitors of the RNA transcriptase activity of influenza viruses. Further evidence in support of this type of zinc ion chelation through sulphur comes from the observed ^{31}P n.m.r. shifts of bisthiopyrophosphate in the presence of various metal ions. It was of interest to determine whether zinc ions coordinated to the oxygen or sulphur ligands in bis-thiopyrophosphate. ^{31}P n.m.r. measurements (Table 5.4 and Fig. 5.4) show that when magnesium, zinc or cadmium ions were added to a solution of pyrophosphate in triethanolamine buffer at pH 8.2, the pH of the RNA transcriptase assay, only small shifts ($\sim$ 1 p.p.m.) of the ^{31}P signal occurred, presumably due to the formation of complexes with the metal ions coordinated to the oxygen atoms of the pyrophosphate.

In the case of bisthiopyrophosphate at pH 8.2, addition of a 'hard' metal ion such as magnesium produced a downfield shift of less than 2 p.p.m., addition of softer metal ions (cadmium or zinc) however, produced large shifts in the ^{31}P n.m.r.

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	Group IA					Group IIA	
	Na		K			Mg	Ca
Biological roles	Charge-carriers and osmotic balance						
Location	Mobile					Semimobile	
Oxidation states	I		I			II	II
Donor atoms preferred	O,$-O^-$		O,$-O^-$			O,$-O^-$	O,$-O^-$
Type of complexes formed	weak					Fairly strong	

						Group IIB
	Mn	Fe	Co	Cu	Mo	Zn
Biological roles	Redox catalysis and enzyme structures					Super-acid catalysis
Location				Static		
Oxidation states	II/III	II/III	II/III	I/II	V/VI	II
Donor atoms preferred	-O	N,$-O^-$	M,$-O^-$	N,$-S^-$	$-S^-$	N,$-S^-$
Type of complexes formed			Strong			Strong

Table 5.5(iii) Complexing characteristics and *in vivo* roles of some metals essential to human life (from Ainscough and Brodie, 1976)

signal of 8-9 p.p.m. Since cadmium ions have been shown to complex with sulphur in nucleoside thiophosphates (Jaffe and Cohn, 1978; Pillai, *et al.*, 1980), the ^{31}P n.m.r. studies suggest that zinc ions coordinate to bisthiopyrophosphate through sulphur. Furthermore, magnesium ions are known to coordinate only to oxygen in phosphorothioates (as determined by x-ray structural analysis for diethyl *O,O*-phosphorothioate (Schwalbe, *et al.*, 1973)) confirming that magnesium ion chelation is unlikely to be of importance in the antiviral action of these compounds.

Magnesium is therefore likely to form a chelate ring of structure I (Fig. 5.5(i)) and cadmium (and therefore zinc) likely to form the probably stronger chelate II.

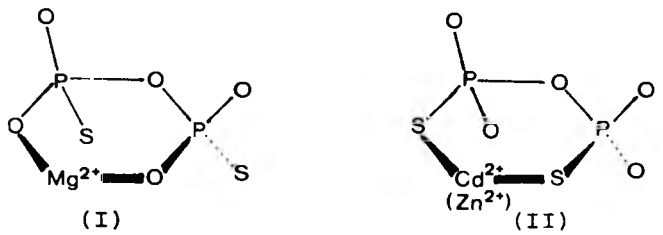


Fig. 5.5(i) Possible chelates of bisthiopyrophosphate with magnesium ion (I) or cadmium and zinc (II).

If this method of coordination also occurs at the essential zinc ion in the transcriptase of influenza virus A, this may account for the difference in inhibitory activity between inorganic pyrophosphate and its thio-analogue.

Thiophosphonates in general do not, however, show any great improvement over the corresponding phosphonates in their antiherpesvirus activity, with similar ID_{50} values being obtained for PAA, PFA, thiophosphonoacetic acid, and thiophosphonoformic acid. Only 5-(thiophosphonomethyl)-1(H)-tetrazole (18) shows improved activity against HSV-1 (KOS) DNA polymerase, inhibiting this enzyme by 50% at a concentration of 90 μM.

The reason for the general increase in antiviral activity of the thiophosphonates against influenza viruses only is unknown. One possibility, however, is differing roles for zinc ions in viral DNA and RNA polymerases. The most likely function of enzyme bound zinc in polymerase enzymes is generally considered to be interaction with the 3'-hydroxyl of the growing end of the DNA and its activation for nucleophilic attack on the α-phosphate of the incoming nucleoside triphosphate (NTP) (Fig. 5.5(ii)) while magnesium ions coordinate via a bidentate β-γ-Mg-NTP complex on the incoming triphosphate (Sigman, *et al.*, 1972; Springgate, *et al.*, 1973; Mildvan and Leob, 1979; Eckstein, 1983).

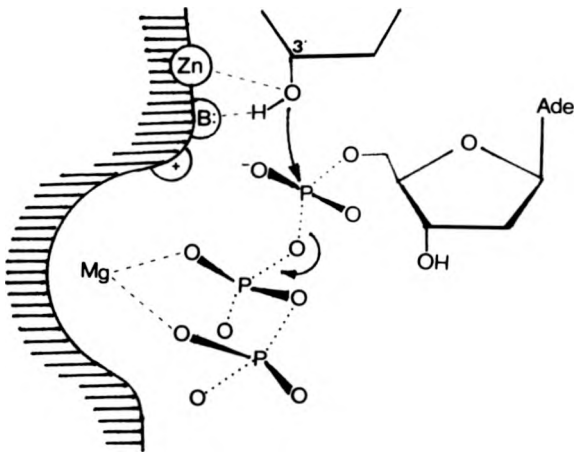
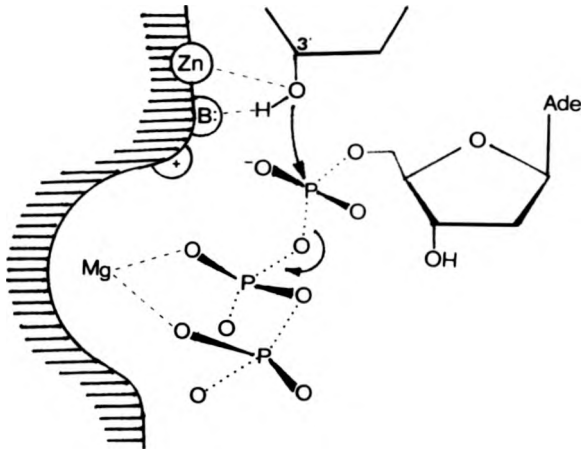


Fig. 5.5(ii) Active site of a polymerase enzyme showing possible role of zinc.

An enzyme bound zinc atom even if merely in close proximity to the pyrophosphate binding site of the polymerase, is likely to have an influence on pyrophosphate-like compounds, particularly those likely to bind strongly to zinc ions such as bisthiopyrophosphate coordinating via two 'soft' sulphur ligands. 5-(Thiophosphonomethyl)-1(H)-tetrazole contains a soft sulphur ligand and a heterocyclic nitrogen ligand, which is considered to be softer than oxygen on the HSAB scale. Indeed zinc in metallo-enzymes is often bound to the heterocyclic nitrogen



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atoms of histidine or cysteinyl sulphur atoms. It should be remembered, however, that the coordination of enzyme bound zinc to atoms of amino acids in the protein is likely to affect its overall hardness or softness (Hutchinson, 1985) and its chelating properties are therefore likely to be altered. Thus, a zinc ion coordinated to oxygen ligands is likely to behave differently towards chelating agents when compared to a zinc ion bound by nitrogen or sulphur. It could reasonably be expected that the stronger the σ-electron donation from the ligand to the metal, the weaker the Lewis acidity of the metal centre. Ligands such as imidazole which are both σ-donors and π-acceptors may 'tailor' the metal ion's Lewis acid character by the degree of π-acceptance. One reason for the differences in sensitivities of various strains of influenza A virus to pyrophosphate analogues (Chapter 4) could be differences in the amino acid residues holding zinc in the enzyme active site, creating zinc ions of slightly different degrees of softness, and therefore different degrees of sensitivity to such zinc chelating agents.

The presence of zinc ions in the pyrophosphate 'well' of polymerase enzymes has not been established, however, it is interesting to note that unlike DNA polymerases, which appear in general to contain only one zinc atom per molecule (Mildvan and Leob, 1979), many RNA polymerases contain multiple zinc ions, despite

single initiation and elongation sites. This suggests multiple roles for zinc ions, including purely structural ones, and could be a reason for the observed greater susceptibility of viral RNA polymerases to zinc chelating pyrophosphate analogues.

One drawback in the development of pyrophosphate analogues as antiviral agents with clinical potential has been the tendency of such compounds to accumulate in bones and teeth, despite the fact that such properties are not known to have any deleterious effects. This is probably due to the ability of pyrophosphate analogues to chelate 'hard' metal ions such as calcium. Since sulphur ligands to not appear to bind with any strength to hard metal ions like magnesium, it is likely that if the oxygen ligands in pyrophosphate analogues can be replaced by softer ligands such as nitrogen and sulphur then the accumulation of these compounds in bones and teeth could be eliminated.

APPENDIX I

SOURCES OF MATERIALS

Adenosine-(3'-5')-guanosine	Sigma (London) Chemical Co., Poole, Dorset, U.K.
Benzylchloromethyl ether	Fluka AG, G.F.R.
Bovine pancreatic DNase I	Sigma (London) Chemical Co., Poole, Dorset, U.K.
Calf thymus DNA polymerase I	Pharmacia Fine Chemicals, Uppsala, Sweden
Cellulose F_{254} TLC plates	E. Merck, Darmstadt, G.F.R.
Chromatography paper (3MM)	Whatman Ltd., Maidstone, Kent, U.K.
DEAE Cellulose DE52	Whatman Ltd., U.K.
DEAE Sephadex A-25	Pharmacia Fine Chemicals, Uppsala, Sweden
DMEM (X10)	Flow Laboratories Ltd., Irvine, Ayrshire, U.K.
Dowex 50, W, 1	Sigma (London) Chemical Co., Poole, Dorset, U.K.
GF/C discs (2.5 cm)	Whatman, Ltd., U.K.
L-Glutamine	Flow Laboratories Ltd., U.K.
$^{3}H_2O$	Amersham International PLC Amersham, Bucks., U.K.

MDCK cells	Flow Laboratories Ltd., U.K.
NCS	Flow Laboratories Ltd., U.K.
NEAA	Flow Laboratories Ltd., U.K.
Penicillin/Streptomycin	Flow Laboratories Ltd., U.K.
Phosphonoacetic acid	Sigma (London) Chemical Co., Poole, Dorset, U.K.
Phosphonoformic acid	Sigma (London) Chemical Co., Poole, Dorset, U.K.
Sephadex G-10	Pharmacia Fine Chemicals, Uppsala, Sweden
Silica gel 60 (230-400 mesh)	E. Merck, G.F.R.
[Methyl-H3]thymidine 5'-triphosphate	Amersham International PLC, Amersham, Bucks, U.K.
[5-H3]Uridine 5'-triphosphate	Amersham International PLC, Amersham, Bucks, U.K.
Vero cells	Flow Laboratories Ltd., Irvine, Ayrshire, U.K.
Zinc chloride (Spectrosol)	B.D.H. Chemicals Ltd., Poole, Dorset, U.K.

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Determining the Stability Constant of a Metal Complex by Gel Chromatography

Let us consider the formation of a 1:1 metal complex, ML, between a metal ion, M, and a ligand, L, according to eqn. (1)

$$M + L \rightleftharpoons ML \tag{1}$$

For the sake of simplicity the charge on each species is not presented. The concentration stability constant, K, of the metal complex to be determined is given by

$$K = \frac{[ML]}{[M][L]} \tag{2}$$

in which [ ] represents the molar concentrations of M, L, and ML.

Various physiochemical methods, such as spectrophotometry, potentiometry, ion exchange methods, solvent extraction methods, solubility methods, etc., have usually been employed to determine the concentration or the activity of M, L, or ML (*1, 2*). If one of these activities or concentrations is experimentally determined, the other two can be calculated, which enables us to evaluate the stability constant of the metal complex. For example, if [ML] is determined spectrophotometrically, [M] and [L] will then be calculated by

$$[M] = [M]_t - [ML] \tag{3}$$

$$[L] = [L]_t - [ML] \tag{4}$$

in which $[M]_t$ and $[L]_t$, the respective total concentrations of metal and ligand, are known.

In this paper we describe the principle of a new technique that is based on a gel chromatographic method (*3–7*) and has been increasingly applied to the characterization of metal-ligand binding in the fields of inorganic (*8*), bioinorganic (*9–17*), and environmental (*18*) chemistry. This chromatographic method has an advantageous and unique characteristic over the conventional static (batchwise) equilibrium methods, in that [M] can be kept at a desired and predetermined value, $[M]_0$, throughout the experiment. In other words, the equilibrium solution being examined is "buffered" with respect to metal ion, as well as with respect to hydrogen ion, and [ML] and [L] are then dynamically adjusted to be in equilibrium with $[M]_0$ through the process of their chromatographic migration. Therefore, [M] is not required to be analyzed experimentally and one can evaluate the stability constant of the metal complex according to eqn., (5) by the measurement of [ML] that is in equilibrium with $[M]_0$.

$$K = \frac{[ML]}{[M]_0([L]_t - [ML])} \tag{5}$$

in which $[M]_0$ and $[L]_t$ are known. The only undetermined value, [ML], can be calculated by

$$[ML] = [M]_t - [M]_0 = a\,(A_{ob} - A_{M_0}) = a\,A_{ob} - b \tag{6}$$

where A_{ob} is the observed absorbance, that is the sum of the respective absorbances, A_{M_0} and A_{ML}, due to free metal ion and metal complex. a and b are constants. Equations (5) and (6) indicate that [ML] or K can be easily determined by measuring A_{ob} without any complicated analysis of the observed results. The atomic absorption method has been extensively and successfully employed to obtain reproducible results (*8, 10, 18*). Spectrophotometric methods are powerful if the absorption due to free ligand is negligible. The terms A_{ob} and A_{M_0} in eqn. (6) can also be translated into any kind of physicochemical responses of ML and M, such as are based on radioactivity measurement (*18*).

The most important point in practice of this approach is how to keep the concentration of free metal ion at a constant level during the equilibrium experiment. It seems very difficult or almost impossible to maintain [M] constant by the conventional static methods in which [M] is usually allowed to be dependent on $[M]_t$ and $[L]_t$ as indicated in eqns. (3) and (4). As will be mentioned below, however, a simple way to answer this requirement is by use of a gel chromatographic method which was first applied by Hummel and Dreyer (*3*) to the binding of 2′-cytidilic acid with ribonuclease and has subsequently been employed for estimation of the stability constants of various metal complexes (*6, 9*), with some progressive modification in practical procedure (*8, 11*). This technique enables us to observe directly the formation of ML complex that is in equilibrium with metal ion of the specified concentration, $[M]_0$.

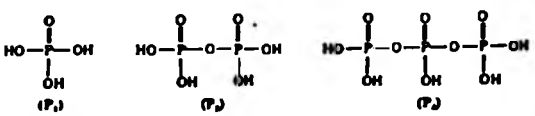
Gel Chromatography of Metal Ions and Ligands

Before we deal with the detailed discussion on the gel chromatographic determination of the stability constant of metal complex let us review briefly the gel chromatographic behavior of two constituents, a metal ion and a ligand (*6, 19*).

Gel chromatography is a form of liquid chromatography capable of separating solute molecules according to their size, and its basic principle has been explained in terms of sieving effect or steric exclusion (*4–7*). The separation of the components in a sample solution is usually carried out on a column packed with a gel or other porous material. It is a general elution pattern, though not always if there are side effects such as adsorption, that the solute molecules are eluted in the order of decreasing molecular size.

Although it was first applied mostly to the separation of very small molecules from very large ones, e.g., desalting from proteins, it has progressively been demonstrated to be widely applicable to the separation of not only a mixture of macromolecules but also a mixture of lower-molecular-weight species as small as hydrated metal ions. For example, the elution volumes of alkaline earth metal ions on a Sephadex G-15 column (Pharmacia Fine Chemicals AB) increased in the order, Mg < Ca ≈ Sr < Ba, which could be qualitatively correlated with the reverse sequence of the radii of these hydrated metal ions, i.e., Mg (4.28 Å) > Ca (4.12 Å) = Sr (4.12 Å) > Ba (4.04 Å) (*20*).

A more pronounced effect of steric exclusion has been shown for the elution behavior of inorganic polyphosphate anions with different degrees of polymerization (*6, 21*). For example, the elution volumes of orthophosphate (P_1), diphosphate (P_2), triphosphate (P_3), tetraphosphate (P_4), and more high polymers (P_n) decreased in the order $P_1 > P_2 > P_3 > P_4 > P_n$. A mixture of P_1, P_2, and P_3, shown below for their acids, can be easily and completely separated from each other.



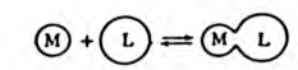
For the quantitative expression, the elution volume of a solute molecule, V_e, is represented in terms of some column parameters (*6*). For very large molecules that are completely excluded from the gel pores, V_e is equal to the interstitial volume of the column, V_0. For very small molecules that can penetrate into all parts of the gel pores, V_e is equal to the total liquid volume of the column, $V_i + V_0$. For molecules of intermediate size, the elution volume is given by

$$V_e = V_0 + K_d V_i \tag{7}$$

in which K_d is a distribution coefficient that depends on the molecular size and lies in the range, $0 \leqq K_d \leqq 1$.

Gel Chromatography of Metal Complex

In eqn. (8) a complexation reaction is shown schematically to illustrate the difference in size among a metal ion, a ligand, and a metal complex (*6, 21*)



(8)

in which the circles represent the sizes of the free metal ion, the free ligand, and the metal-ligand complex. It is assumed that if the size of the free metal ion is relatively very small compared with that of the free ligand, the size of the metal complex may be primarily determined by the contribution from the size of the ligand. In such a situation, the metal complex is expected to appear at or near the elution position of the free ligand. This speculation seems reasonable to a first approximation for polyatomic ligands such as inorganic polyphosphates, EDTA, nucleotides, proteins, etc. The elution volumes of magnesium ion and magnesium polyphosphate complexes, for example, decrease in the order, $Mg > MgP_2 > MgP_3 > MgP_4 > MgP_n$, which is in accord with the prediction from the increasing order in the ionic sizes, $P_2 < P_3 < P_4 < P_n$, or the decreasing order in the elution volumes, $P_2 > P_3 > P_4 > P_n$, of the corresponding polyphosphate anions (*8*). The metal complex in some cases tends to be eluted later than the free ligand (*6, 22, 23*), for which no satisfactory explanation has been given. It is conceivable that the size reduction of the metal complex, in contrast to the expectation from the molecular weight basis, may be caused by such effects as the chelation and the reduction in charge of the hydration layer.

It should be noted that if the ML complex is eluted with an eluent containing neither M or L, it will dissociate successively into its constituents during the passage through the column to give a complicated elution profile of M, L, and ML, depending on both the stability constant and kinetic factors (*19*). On the other hand, if an eluent contains a constant concentration of M and the complexation equilibrium is established rapidly so as not to permit the separation of ML and L, a well-defined peak of ML accompanied by L is expected to appear at the elution position that corresponds to the weighted average of the elution volumes of ML and L.

Determination of Stability Constant

For the sake of clarity in expression, the formation of a magnesium complex, MgL, will be hereafter presented in interpreting how one can determine the stability constant of a metal complex (*6, 8, 19*).

A gel chromatographic column packed with a tightly cross-linked gel, such as Sephadex G-10, G-15, and G-25 (Pharmacia Fine Chemicals AB) is pre-equilibrated with a buffered eluent containing a known concentration of magnesium ion, $[Mg]_0$. A known amount of a ligand, L, is dissolved in a solution of magnesium ion to prepare a sample solution. It is an important prerequisite that the total magnesium concentration in the sample be exactly the same as that in the eluent, if a Hummel and Dreyer pattern (Fig. 1) is to be obtained. This means that the concentration of free magnesium ion in the sample solution is reduced to a level lower than $[Mg]_0$ by an amount corresponding to the MgL complex formed.

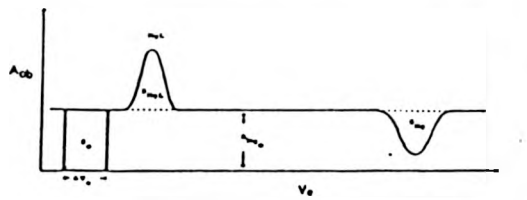


Figure 1. A schematic representation of Hummel and Dreyer pattern. According to eqn. (6) $[Mg]_0 = a\ A_{Mg_0}$

An aliquot of this sample solution is applied to the pre-equilibrated column and then eluted with the eluent used to preequilibrate the column. The MgL complex, along with L that is in a state of dynamic equilibrium with ML, migrates down the column more rapidly than the free magnesium ion so as to come out from the zone of magnesium deficiency. L and ML continue their travel through the pre-equilibrated column to be in contact with fresh free magnesium ions in the subsequent plates to form more MgL until a steady state is reached in equilibrium with $[Mg]_0$ that is the concentration of free magnesium ion in the mobile phase of the pre-equilibrated column. The resulting elution profile is schematically shown in Figure 1. The absorbance, A_{ob}, corresponding to the total magnesium concentration, $[Mg]_t = [Mg] + [MgL]$, is assumed to be monitored by atomic absorption methods and plotted against the elution volume (see eqn. (7)).

When MgL along with L emerges faster, the total concentration of magnesium in the effluent rises above the base line level to form a positive peak of MgL. Behind the MgL peak, A_{ob} continues to be constant and then decreases, at the elution position of free magnesium ion, to below the base line level to form a negative peak that corresponds to the amount of magnesium consumed to form MgL. The appearance of a pair of positive and negative peaks in the elution profile therefore provides a criterion of binding of M and L. It is evident that the height of the horizontal base line corresponds to $[Mg]_0$. The concentration level of free magnesium in the zone of MgL complex has been interpreted to be equal to that in the horizontal region, provided that the sample concentration is reasonably low in comparison with the concentration of background electrolyte in the eluent (*8, 11*). In other words, the peak area of MgL above the base line, S_{MgL}, is a direct measure of the total amount of MgL complex that is in equilibrium with free magnesium whose concentration is $[Mg]_0$. It is also noted that S_{MgL} should be equal to the area of the negative peak, S_{Mg}. The peak area S_{MgL} or S_{Mg} can be easily translated into the amount of MgL, Q_{MgL}, on the basis of the standardized area, S_0, that is regarded as an internal standard to be calibrated against the known amount of magnesium, $\Delta V_e [Mg]_0$. Equation (5) thus can be rewritten as follows to simplify the calculation of the stability constant

$$K = \frac{Q_{MgL}}{[Mg]_0 (Q_{L_t} - Q_{MgL})} \tag{9}$$

where Q_{L_t} represents the total amount of L applied.

The ratio of Q_{MgL} to Q_{L_t} is designated as $\bar{n}$ and is correlated to K and $[Mg]_0$ by

$$\bar{n} = \frac{Q_{MgL}}{Q_{L_t}} = \frac{K [Mg]_0}{1 + K [Mg]_0} \tag{10}$$

The Hummel and Dreyer method in Figure 1 has a great advantage that Q_{MgL} can be estimated as well from S_{Mg} even when S_{MgL} can not be quantitatively determined by the spectrophotometric method owing to the interference by L that is also at the elution position of MgL. The exact measurement of S_{Mg}, however, is often difficult because the negative peak of free metal ions tends in general to broaden

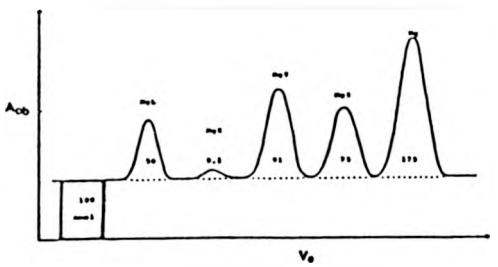


Figure 2. A schematic representation of an elution profile for a mixed solution of magnesium ion and four ligands, i.e., L, X, Y, and Z.

markedly if the metal ions are susceptible to adsorption. Another disadvantage of the S_{M_g} measurement is that S_{M_g} can not be directly translated into Q_{M_gL} if more than one ligand is contained in a sample to form various metal complexes (Fig. 2). To minimize the analytical error resulting from the peak area measurement it is recommended that the relatively well-defined peak of MgL complex be measured by employment of specific methods such as atomic absorption (*8*) and, for metals such as Zn-65, radioactivity measurement (*18*). In such instances the preparation of a sample solution becomes more convenient because magnesium concentration in the sample solution need not be the same as that in the eluent. It is in reasonable excess so as to obtain a positive peak at the elution position of free magnesium ion (Fig. 2), in contrast to the negative peak in Figure 1.

A schematic profile to be expected is shown in Figure 2 when a mixed solution (1 ml) containing each 100 nmole of four ligands, i.e., L, X, Y, and Z that are different in size from each other, is assumed to be eluted with an eluent; $[Mg]_0 = 10^{-5}\ M$. The sample solution also contains 410 nmole of total magnesium that is 400 nmole in excess of that in 1 ml of the eluent. If the stability constants of four magnesium complexes are given as 1.0×10^5 for MgL, 1.0×10^4 for MgX, 1.0×10^6 for MgY, and 3.0×10^5 for MgZ, one can expect the amounts of the respective magnesium complexes, Q_{M_gL}, Q_{M_gX}, Q_{M_gY}, and Q_{M_gZ}, that are numerically (in nmole) shown in Figure 2 for four peaks of magnesium complexes, as well as for a positive peak of free magnesium ion. In other words, one can easily calculate the stability constant, for example, of MgZ complex according to eqns. (9) and (11), if Q_{M_gZ} is given.

$$K = \frac{75}{1.0 \times 10^{-5}\,(100 - 75)} = 3.0 \times 10^5 \qquad (11)$$

There is an example of the exercise in calculation of this class which is based on the peak area measurement on the chart of a magnesium diphosphate complex (*8*) whose log K value obtained by other static methods (*24*) has been reported to be 5.42.

Numerical values in Figure 2 also indicate that the peaks for magnesium complexes increase at the expense of the peak of free magnesium ion. It should be noted, however, that the amount of each magnesium complex is dependent on $[Mg]_0$ but does not depend on both the amount of free magnesium ion in the sample or the peak area of free magnesium ion and the presence of other ligands. Therefore, Q_{M_gL} values obtained by both methods in Figures 1 and 2 should be, in principle, equal to each other, so long as $[Mg]_0$ is the same.

According to eqn. (10) Q_{M_gL} or $\bar{n}$ depends on $[Mg]_0$. If $[Mg]_0$ is lowered from $1.0 \times 10^{-5}\ M$ to $1.0 \times 10^{-6}\ M$, the values of Q_{M_gL}, Q_{M_gX}, Q_{M_gY}, and Q_{M_gZ} will accordingly decrease from 50, 9.1, 91, and 75 to 9.1, 1.0, 50, and 23, respectively, to result in the increase in the amount of free magnesium ion from 175 to 317.

The ligand concentration is usually required to be very low, in comparison to the concentration of background electrolyte, to avoid the complexity caused by the Donnan effect (*11, 13, 25*). In the accurate analysis of such a dilute sample solution it is important to know how to choose $[Mg]_0$ for sensitive detection and how to minimize the analytical error. It is very convenient in practical use to remember that, if $K\,[Mg]_0 = 1$, 50% of the total ligand can be detected as MgL and the relative error in estimating K can be predicted to be twice the relative error in the measurement of Q_{M_gL}, which is acceptable. On the other hand, at $K[Mg]_0 = 10$, the advantage that about 90% of the total ligand can be detected as MgL can not overcome the drawback of the unfavorable relative error in K that amounts to approximately ten times that in Q_{M_gL}.

Many investigations have been reported on the binding of more than one metal to a ligand (*12–18*), including the competitive or cooperative binding of more than one kind of metal (*11, 26*). In order to avoid the complexity in the description of the principle, however, the information from these interesting reports can not be presented in this paper.

The gel chromatographic method has application from the analytical viewpoint. It is evident from eqn. (10) that Q_{M_gL} or S_{M_gL} can be calibrated against Q_{L_0} to be determined. For example, gel chromatographic technqiue combined with an atomic absorption flow detector has been successfully applied to the automatic and sensitive analysis of a mixture of various polyphosphates (*8*). This method will be in principle applicable to the detection of any kinds of ligands which combine with a given metal ion that can be sensitively detected by atomic absorption or other selective methods.

Questions on the Mechanism

We have received many questions about this new technique from those who are familiar with the conventional static equilibrium methods in determining the stability constant. Most of these questions are based on a doubt as to whether or not the equilibrium we can observe in such a dynamic transport process can be regarded as the same as that in a static equilibrium system. One of the important questions is how one can realize that MgL and L are in equilibrium with $[Mg]_0$. As has been pointed out in the original paper of Hummel and Dreyer (*3*) the establishment of a steady state in equilibrium with $[Mg]_0$ is visually reflected on the appearance of a base line level corresponding to $[Mg]_0$ between the peaks of MgL and Mg.

The second question is whether or not this technique is also applicable, as shown in Figure 2, to metal complexes that are not large enough to be completely excluded from the gel phase. This doubt may arise, unfortunately, from the confusing description in the literature by some gel chromatographers who stressed the preferable use of the gel type that permits the complete exclusion of MgL and L. As has been noted by Determann and Brewer (*5*), it is not necessary for the ligand to be excluded completely from the gel phase. A prerequisite is that the degree of separation between the peaks of MgL and Mg be good so as to permit the appearance of a base line corresponding to $[Mg]_0$ between two peaks. This technique, for example, can be successfully applied to the relatively small ligands such as EDTA and diphosphate anions whose K_d values in eqn. (7) are not zero (*8, 23*).

The third question is about the effect of the stationary phase on the complexation equilibrium, which is related in a complicated manner to the above two questions. This question seems out of consideration for MgL and L, whose K_d values are zero, and which is present only in the mobile phase. To be considered is the case for smaller MgL and L that are distributed not only in the mobile phase but also, in part, in the stationary phase. In order to make the answer as simple as possible, let us remember that, according to a random go-and-stop concept in chromatography (*27*), a solute molecule migrates down the column only when it is in the mobile phase, while its down-stream motion in the stationary phase is halted. This means that the effluent we can analyze contains only the equilibrium components in the mobile phase at the last plate of the column. The stationary gel phase plays an important

role in separating MgL and Mg to make a steady state in equilibrium but does not affect the complexation equilibrium in the mobile phase that we can determine. The interstitial mobile phase (space) between gel particles may be regarded as a vessel in which a static equilibrium experiment is carried out.

The author wishes to express his hearty thanks to Professor Shigeru Ohashi for his encouragement during this work.

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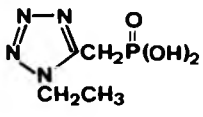
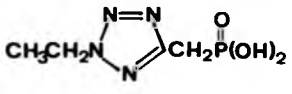
APPENDIX III

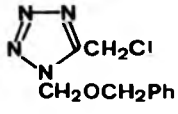
DETERMINATION OF THE STRUCUTRE OF N-ALKYLATED TETRAZOLES BY THE MEASUREMENT OF NUCLEAR OVERHAUSER EFFECTS



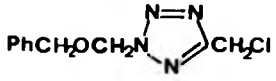
1a



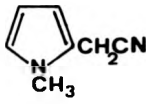
1b



2a



2b



3

Fig. A.III(i)

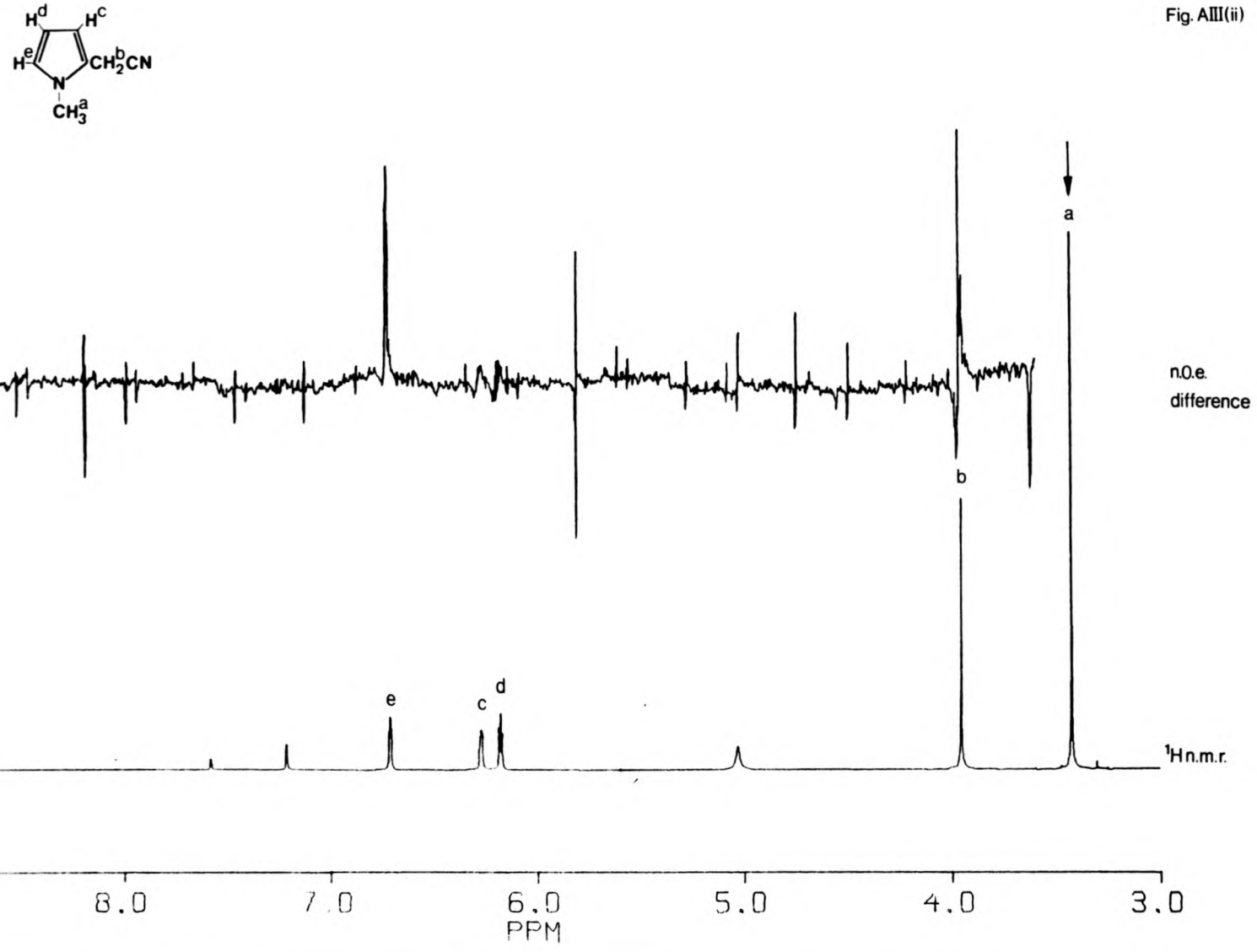
In order to determine whether alkylation of the tetrazole ring occurs at the N-1 or N-2 position, and therefore differentiate between structures (1a)-(1b) and (2a)-(2b), nuclear Overhauser enhancement effects were studied.

Nuclear Overhauser enhancement measurements were carried out at 400 MHz of a D_2O solution of (1) and $CDCl_3$ solution of (2). When either the signals due to the protons of the methylene group attached to

nitrogen ((1), 4.6 p.p.m.; (2), 5.9 p.p.m.), phosphorus ((1), 3.4 p.p.m.) or chlorine ((2), 4.65 p.p.m.) were irradiated for 1.5 s before accumulation of the ^{1}H n.m.r. spectrum, no n.O.e. enhancement of any signals was detected.

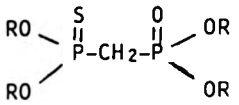
When the signal due to the protons of the N-methyl group (3.45 p.p.m.) of 1-methyl-2-pyrroleaceto-nitrile (3) (400 MHz, d_5-pyridine solution) was irradiated under the same conditions, significant n.O.e. enhancement of the signals at 4.0 ($-CH_2CN$) and 6.7 p.p.m. (ring proton alpha to the N-1 nitrogen atom) occurred (Fig. A.III(ii)).

The n.O.e. results therefore suggest that structures (1b) and (2b) are correct, with ring alkylation occurring at the N-2 position of the tetrazole ring, since significant n.O.e. enhancement occurs only when an N-methyl substituent in the 1-position of (3) is irradiated.


H^d
H^c
H^e
CH2^b CN
N
CH3^a
Fig. AIII(ii)
a
b
n.O.e.
difference
e
c
d
1H n.m.r.
8.0
7.0
6.0
5.0
4.0
3.0
PPM

APPENDIX IV

EXPERIMENTAL



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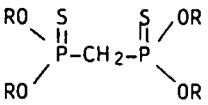
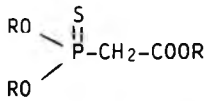
R = Me_3Si 3
R = $NHEt_3$ 7
R = Na 8



R= Et 2
R= Me_3Si 4
R= $NHEt_3$ 9



R= Et 5
R= Me_3Si 6
R= $NHEt_3$ 10

^1H NMR spectra were recorded with a 60 MHz varian spectrometer (EM 360). A Bruker WP 80 SY spectrometer was used for ^{13}C NMR (internal TMS) and $^{31}PNMR$ (H_3PO_4 as external standard). δ are given in ppm and J in Hz.

Starting materials

Diethyl methanethiophosphonate was prepared by a Michaelis-Becker reaction (1) from diethylthiophosphite and iodomethane. Diethylthiophosphite was obtained by sulfuration (H_2S in pyridine) of commercial diethylchlorophosphite according to (2).

Diethyl (O,O-diethyl thiophosphono)- methylphosphonate 1

To a stirred solution of lithium diisopropylamide prepared from 4.78 ml (33.9 mmol) of diisopropylamine and 25 ml of a solution (1.3 M in hexane) of BuLi (32.4 mmol) and 50 ml of THF maintained under N_2 atmosphere and cooled to -78°C, is added dropwise 2.59 g (15.4 mmol) of diethyl methane thiophosphonate. Stirring is maintained fo 15 min. after the end of the addition and then 5.59 g (32.4 mmol) of diethyl chlorophosphite are added dropwise. The solution is allowed to warm to -20°C (3 h) and then poured in a saturated aqueous NH_4Cl solution, acidified with 5 % aqueous HCl solution and extracted with ether. The ether extracts are washed with brine, dried over sodium sulfate and concentrated under vacuo.

The residue (4.51 g) is distilled under reduced pressure to yield 2.95 g (63 %) of a colourless liquid bp : 103-105° (0.01 mm)

^{1}H NMR (CCl_4) : 1.33 (t, J= 7, 12 H), 2.60 (dd, $^{2}J_{HP(O)}$ = 20.6, $^{2}J_{HP(S)}$= 18,8 , 2H) 4.10 (dq, J_{HH}= 7, $^{3}J_{HP}$= 7,8 H).

^{13}C NMR ($CDCl_3$) : 16.25 (2d, $\underline{C}H_3$-), 3.49 (dd, $J_{P(O)C}$ = 135.94, $J_{P(S)-C}$ = 107.23, -$\underline{C}H_2$-), 62.71 (2d, CH_3-$\underline{C}H_2$-O-).

^{31}P NMR ($CDCl_3$) : 83.16 [d, P(S)]; 17.75 [d, P(O)] ; J_{PP}= 7

Analysis : Calc. for $C_9H_{22}O_5P_2S$: C 35.52 H, 7.29 O, 26.29 S, 10.54 ; P, 20.39. Found : C, 35.26 ; H, 7.35 ; O, 26,21 ; S, 10.00.

0,0,0'0'-tetraethyl methylene bis-thiophosphonate 2

This compound is prepared by U.V. induced Michaelis Becker-reaction from diethylthiophosphite and dibromomethane according to the described method (3).

^{1}H NMR (CCl_4) : 1.32 (t, J= 7, 12 H) ; 2.84 (t, $^{2}J_{HP}$= 18, 2H) ; 4.1 (dq, J_{HH}= 7, $^{3}J_{HP}$= 10, 8H).

^{13}C NMR ($CDCl_3$) 16.12 (quint. $\underline{C}H_3$-), 42.55 (t, J_{P-C} = 106.29, -CH_2-), 62.91 (quint. CH_3-$\underline{C}H_2$-O).

^{31}P NMR ($CDCl_3$) : 82.29.

Bis- (trimethylsilyl) [0,0-bis-(trimethylsilyl) thiophosphono]-methylphosphonate 3

Bisphosphonate 1 (2 mmol) is warmed at 100°C with (10 mmol) of iodotrimethylsilane for 48 h. The dark brown mixture is distilled bp : 110-112° (0.03 m), 0.645 g of 4 is obtained as a liquid (still coloured by some iodine).

^{1}H NMR ($CDCl_3$) : 0.25 (s, 36H) ; 2.60 (dd, $^{2}J_{HP(O)}$= 21, $^{2}J_{HP(S)}$ = 18.5, 2H)

^{31}P NMR ($CDCl_3$) : 50.05 [P(S)] , 0.21 [P(O)].

0,0,0'0'-tetrakis-(trimethylsilyl) methylene bis-thiophosphonate 4

Bisphosphonate 2 (2 mmol) is warmed at 100° with 1,70 ml (12 mmol) of iodotrimethylsilane for 10 days. Distillation of the dark brown mixture give 0,532 g of a liquid (containing traces of iodine) bp : 125.130°C (0.03 m).

^{1}H NMR ($CDCl_3$) : 0.25 (s, 36 H), 2.88 (t, $^{2}J_{HP}$= 18.5, 2H)

^{31}P NMR ($CDCl_3$) : 56.64.

Ethyl (0,0-diethyl thiophosphono)-acetate 5 (4)

To a stirred suspension of sodium hydride (0.6 g, 0.015 mol) in ether (20 ml) are added dropwise 2.04 ml (0.015 mol) of diethylthiophosphite (evolution of hydrogen is observed). The mixture is refluxed for 30 min. and Then cooled to 0°C. Ethyl chloroacetate (2.67 ml, 0.025 mol) is slowly added and the mixture is allowed to react at room temperature for 48 h. A minimum of water is added for the dissolution of the salts and after decantation the etheral layer is dried over sodium sulfate. After evaporation of the solvent the residue is distilled. 1.65 g (46 %) of a colourless liquid bp : 95°C (0.01 mm).

^{1}H NMR (CCl_4) δ : 1.3 ($\sim$ t, J= 7, 9H) ; 3.0 (d, J_{HP}= 20, 2H) ; 4.13 (m, 6H).

^{31}P NMR ($CDCl_3$) δ : 84.91.

Trimethylsilyl (0,0-trimethylsilyl thiophosphono)-acetate 6

Thiophosphonoacetate 5 (0.720 g, 3 mmol) is warmed at 100° with (1.70 ml, 12 mmol) of iodotrimethylsilane for 72 h. The brown solution is distilled under reduced pressure. 0.480 g of a liquid bp : 108-110 (0.03 mm) is recovered.

^{1}H NMR ($CDCl_3$) δ : 0.28 (s, 27 H), 3.12 (d, J= 19.5, 2H)

^{31}P NMR ($CDCl_3$) δ: 56.92.

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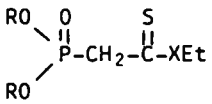
EXPERIMENTAL



R= Et X= O 1
R= Et X= S 2
R= H X= O 3
R= H X= S 4

O-Ethyl (diethylphosphono)thioacetate 1

1 was prepared according to (1)

Diethyl cyanomethylphosphonate (8.85 g, 0.05 mol) and ethanol (4.6 g, 0.1 mol) are dissolved in 100 ml of benzene. The solution cooled to 5°C is saturated with gazeous chlorhydric acid and allowed to react 16 h. Benzene is evaporated and the residuetaken up by 80 ml of pyridine. The solution cooled to 0°C is saturated by hydrogen sulfide (2 h) and allowed to react 16 h. The mixture is poured in 400 ml of an aqueous solution of chlorhydric acid (20 %) and extracted with ether. The extracts are washed several times with the chlorhydric acid solution, water and brine then dried over sodium sulfate. After evaporation of the solvent, the residu is distilled under reduced pressure. 9.37 g of a pale yellow liquid is obtained, bp : 90-91°C (0.08 mm), yield : 78 %.
^{1}H NMR (CCl_4) : 1.2 to 1.6 (3 t, 9H) ; 3.42 (d, $^2J_{PH}$ = 23, 2H) ; 4.03 (dq, $^3J_{HP}$= 7, J_{HH}= 6.5, 4H) ; 4.55 (q, J= 6.5, 2H).

Ethyl (diethylphosphono) dithioacetate 2

Prepared in a 62 % yield by the method described above, ethanethiol being used instead of ethanol. [2 has been prepared recently by another way (2)]
^{1}H NMR (CCl_4) : 1.33 (~t, J= 6.5, 9H) ; 3.23 (q, J= 6.5, 2H) ; 3.62 (d, $^2J_{HP}$= 23, 2H) ; 4.10 (dq, $^3J_{HP}$= 7, J_{HH}= 6.5, 4H).

O-Ethyl phosphono - thioacetate 3

Iodotrimethylsilane (0.427 ml, 3 mmol) is added dropwise to a solution of thioacetate 1 (0.256 g, 1 mmol) in 4 ml of methylene chloride cooled to 0°C. After 2 h at this temperature, 2 ml of water is added and the mixture is stirred for 20 mn. After decantation the aqueous phase is saturated with NaCl and extracted several timeswith ether. The etheral extracts are dried over sodium sulfate and after evaporation of the solvent the residu is cristallized in a carbon tetrachloride - ethyl acetate mixture. 0.118 g (64 %) of pale yellow needles, mp : 103°C, are obtained.

^{1}H NMR (acetone d^6) : 1.38 (t, J= 7, 3H) ; 3.55 (d, $^2J_{HP}$ = 23, 2H) ; 4.53 (q, J_{HP}= 7, 2H).
Analysis (calc; for $C_4H_9O_4PS$) : C, 26.09 ; H, 4.92 ; S, 17.40
Found : C, 28.88 ; H, 4.90 ; S, 16.83.

Ethyl phosphono - dithioacetate 4

Prepared as above. 155 mg (77 %) of yellow cristals mp : 122°C are obtained
^{1}H NMR (acetone d^6) : 1.3 (t, J= 7, 3H) ; 3.26 (q, $^3J_{HP}$= 7, 2H) ; 3.83 (d, $^2J_{HP}$= 23, 2H) ;
Analysis(calc. for $C_4H_9O_3PS_2$) : C, 23.86 ; H, 4.51 ; S : 31.86. Found : C, 24.04 H, 4.14 ; S, 30.85.

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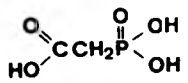
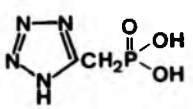
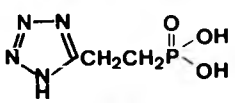
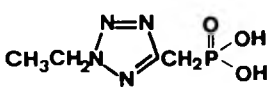
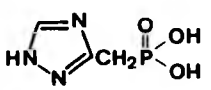
LIST OF COMPOUNDS STUDIED AND THEIR STRUCTURES

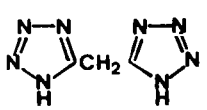
PAA 

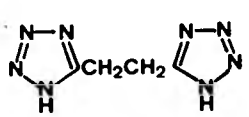
1 

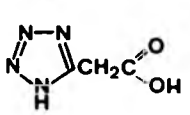
2 

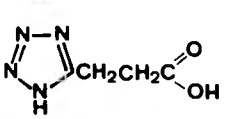
3 

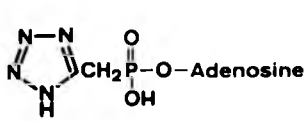
4 

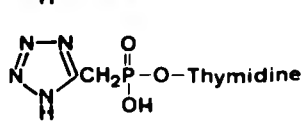
5 

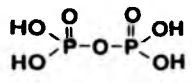
6 

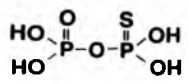
7 

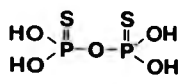
8 

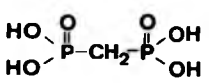
9 

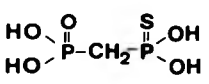
10 

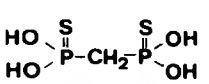
11 

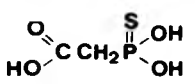
12 

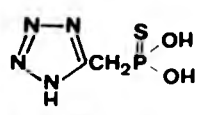
13 

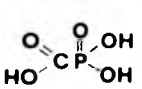
14 

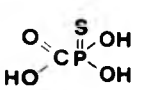
15 

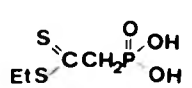
16 

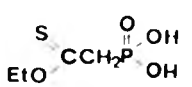
17 

18 

PFA 

19 

21 

20 

THE ANTIVIRAL ACTIVITY OF TETRAZOLE PHOSPHONIC ACIDS AND THEIR ANALOGUES

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ABSTRACT

5-(Phosphonomethyl)-1H-tetrazole and a number of related tetrazoles have been prepared and their effects on the replication of Herpes Simplex Viruses-1 and -2 have been investigated as well as their abilities to inhibit the DNA polymerases induced by these viruses and the RNA transcriptase activity of influenza virus A. Contrary to an earlier report, 5-(phosphonomethyl)-1H- tetrazole was not an efficient inhibitor of the replication of HSV-1 and HSV-2 in tissue culture. Analogues of 5-(phosphonomethyl)-1H-tetrazole were also devoid of significant antiviral activity. Only 5-(phosphonomethyl)-1H-tetrazole and 5-(thiophosphonomethyl)-1H-tetrazole inhibited the influenza virus transcriptase, and both were more effective as inhibitors than phosphonoacetic acid under the same conditions. The DNA polymerases induced by HSV-1 and HSV-2 were inhibited slightly by 5-(phosphonomethyl)-1H-tetrazole and to a lesser extent by its N-ethyl analogue and 3-(phosphonomethyl)-1H-1,2,4-triazole, although none of these compounds were as effective as phosphonoacetic acid. 5-(Thiophosphonomethyl)-1H-tetrazole was a better inhibitor of the DNA polymerase induced by HSV-1 than 5-(phosphonomethyl)-1H-tetrazole.

INTRODUCTION

Analogues of inorganic pyrophosphate, e.g., phosphonoacetic[1] or phosphonoformic[2] acids are selective inhibitors of the DNA polymerases of several viruses of the Herpes family and act directly on the enzymes rather than by being metabolised to an active form such as a nucleoside triphosphate analogue. The RNA transcriptase activity of influenza virus A can also be inhibited by pyrophosphate analogues[3] and, for example, in the presence of phosphonoformic acid initiation of mRNA synthesis occurs but chain elongation does not take place[4]. We believe that the effectiveness of a pyrophosphate analogue as an inhibtor of influenza RNA transcriptase is related to its ability to chelate metal ions, notably zinc[3,5] as the latter is probably an important component of the active site of the transcriptase. We have recently examined other pyrophosphate analogues.

e.g., mono- and bis-thiopyrophosphate as inhibitors of herpes DNA polymerase and the replication of influenza virus in MDCK cells[6], and now wish to report our observations on the antiviral properties of a number of heterocyclic phosphonic acids and their analogues.

It is well known that 1H-tetrazole can be regarded as an isostere of the carboxylic acid group[7] and amino acid analogues in which the carboxylic acid moiety has been replaced by a tetrazole ring have similar pKa values to their parent compounds. Furthermore, the tetrazole moiety can form stable complexes with metal ions. A recent report[8] that 5-(phosphonomethyl)-1H-tetrazole (1) was more effective than phosphonoacetic acid in inhibiting the replication of HSV-2 prompted us to prepare this compound and a number of related derivatives and to investigate their effects on the replication of HSV-1 and HSV-2 in tissue culture and on the inhibition of the DNA polymerases induced by these viruses. The effect of these compounds on the RNA transcriptase of influenza virus A was also investigated.

MATERIALS AND METHODS

Virus and Enzyme Assays

The HSV-1 strain used was KOS and the HSV-2 strain was 3345. The inhibition of the DNA polymerases induced by these viruses in HeLa cells was carried out by the method of Powell and Purifroy[9]. The effects of heterocyclic phosphonic acids and their analogues on the replication of HSV-1 and HSV-2 were determined by plaque reduction assays. Vero cells (2×10^5 cells per well) were grown to confluence in maintenance medium [Dulbecco's modified Eagle's medium, 10% foetal calf serum, glutamine (4.5 g/l)] in 24 well plates and each well was infected with virus (50 p.f.u. in 0.2 ml). After virus absorption had taken place (1 hour, 4^{o}), the CMC overlay (0.7 ml) was added together with the test compound in maintenance medium (0.1 ml). The plates were then incubated for 3-4 days at 37^{o} in a humidified atmosphere of CO_2 (5%) in air. The number of plaques in each well were then determined after fixing and staining with Giemsa stain. Control wells without test compound showed on average 50 plaques per well.

The influenza virus strain used was A/X49, a cross between A/England/864/75 and A/PR/8/34 with the H3N2 surface antigens of the A/England strain. RNA transcriptase assays and the effect of test

compounds on virus replication in MDCK cells was carried out as previously described[6].

Chemical Procedures

^1H N.m.r. spectra were recorded at 220 MHz and chemical shifts are given in p.p.m. with either tetramethylsilane (for $CDCl_3$ and d_6-DMSO) or sodium 3-(trimethylsilyl)-1-propane sulphonate (for D_2O) as standards. ^{31}P N.m.r. spectra were recorded at 36.44 MHz and chemical shifts are recorded relative to 85% H_3PO_4 (external, downfield shifts positive). Chemical ionisation mass spectra were determined using ammonia as reagent gas as previously described[10]. Triazoles, tetrazoles and their phosphonate esters were purified by silica gel chromatography by the method of Still[11] using the following solvent systems; A, acetone/60-80° petroleum ether (3/7, v/v); B, $CHCl_3$; C, methanol/$CHCl_3$ (1/9, v/v). The determination of pK_d, values related to the strength of binding of zinc ions to phosphonates was by gel filtration[3].

Preparation of Tetrazole Phosphonic Acids

5-(Phosphonomethyl)-1H-tetrazole (1).- 5-(Chloromethyl)-1H-tetrazole was prepared by heating chloroacetonitrile (6.4 ml, 0.1 mol) with dry sodium azide (29.5 g, 0.44 mol) and anhydrous aluminium chloride (13.3 g, 0.1 mol) in dry THF (200 ml) under reflux in a nitrogen atmosphere for 14 hours. The reaction mixture was cooled, acidified with 5 M HCl and then extracted with ethyl acetate (3 x 100 ml). The combined extracts were dried (Na_2SO_4) and the solvent removed *in vacuo*. The residue was recrystallised from $CHCl_3$ to give the chloromethyl tetrazole (9.6 g, 81%), m.p. 85°, lit.[12] 85°, ^1H n.m.r. (d_6 DMSO) δ 5.1 p.p.m., CIMS *m/z* 119 $[M + H]^+$, 136 $[M + NH_4]^+$.

N-(Benzyloxymethyl)-5-(chloromethyl)-tetrazole.- To the chloromethyl tetrazole obtained above (5.0 g, 1 equiv.) in dry dioxan (60 ml) was added gradualy, with stirring, sodium hydride (50% suspension in oil, 2.25 g, 1.1 equiv.). After 30 minutes, benzyl chloromethyl ether (6.0 g, 1 equiv.) was added and the mixture stired for 16 hours at 25°. The reaction was then filtered, the solvent removed from the filtrate *in vacuo* and the residue purified by silica chromatography (solvent A) to give *N*-(benzyloxymethyl)-5-(chloromethyl)-tetrazole (8.6 g, 85%). ^1H n.m.r. ($CDCl_3$) δ 4.65 (2H s), 4.8 (2H s), 5.9 (2H s) and 7.3 p.p.m. (5H

m), CIMS m/z 239 $[M + H]^+$, 256 $[M + NH_4]^+$. *N*-(Benzyloxymethyl)-(chloromethyl)-tetrazole (2.4 g, 0.01 mol) was stirred at 120 for 14 hours in triethyl phosphite (5.0 g, 0.033 mol). After this time, excess triethyl phosphite was removed *in vacuo* and the residue purified by silica chromatography (solvent B) to give the diethyl ester of *N*-(benzyloxymethyl)-5-(phosphonomethyl)-tetrazole (2.2 g, 65%), ^1H n.m.r. ($CDCl_3$) δ 1.3 (6H t, J = 6 Hz), 3.55 (2H d, J = 20 Hz), 4.2 (4H m), 4.65 (2H s), 5.9 (2H s), and 7.3 p.p.m. (5H m), CIMS m/z 341 $[M + H]^+$, 358 $[M + NH_4]^+$.

This diethyl ester was stirred in trifluoroacetic acid (8 ml) for 72 hours at room temperature. Removal of the solvent *in vacuo* afforded diethyl 5-(phosphonomethyl)-1H-tetrazole (1.3 g, 90%) after purification by silica chromatography (solvent C), ^1H n.m.r. ($CDCl_3$) δ 1.35 (6H t, J = 6 Hz), 3.75 (2H d, J = 20 Hz) and 4.256 (4H m, J = 7 Hz), CIMS m/z 221 $[M + H]^+$, 238 $[M + NH_4]^+$.

Diethyl 5-phosphonomethyl)-1H-tetrazole (1.1 g) was stirred for 2 hours at room temperature with bromotrimethylsilane (1.52 g). Evaporation of the silane gave a residue to which excess water was added, the latter was removed by lyophilisation and the addition of water and lyophilisation was repeated several times. The product was purified by chromatography on DEAE-Sephadex A25 (Et_3NH^+ form 1.5 x 45 cm) and elution with a gradient of 0.5-1.0 M Et_3NHHCO_3 (pH 8.4) to give the triethylammonium salt of 5-(phosphonomethyl)-1H-tetrazole which was converted using a Dowex 50 (H^+ form) column into the free acid (615 mg, 75%, m.p. 170° (dec.). ^1H n.m.r. (D_2O) δ 3.5 p.p.m. (d, J = 20 Hz), ^{31}P n.m.r. δ 16.9 p.p.m., Analysis C 14.60, H 3.33, N 33.12, P 18.42%, $C_2H_5N_4O_3P$ requires C 14.64, H 3.07, N 34.15, P 18.88%.

5-(2'-Phosphonoethyl)-1H-tetrazole (2).- This was prepared as described above from 5-(2'-chloroethyl)-1H-tetrazole and was isolated as the disodium salt after chromatography on a Dowex 50 (Na^+ form) column, ^1H n.m.r. (D_2O) δ 2.1 (2H m), 3.2 p.p.m. (2H m). ^{31}P n.m.r. (D_2O) δ 24.7 p.p.m., Analysis C 15.97, H 3.11, N 26.21, P 13.88%, $C_3H_5N_4O_3PNa_2$ requires C 16.20, H 2.30, N 25.20, P 13.95%.

5-(Thiophosphonomethyl)-1H-tetrazole (3).- Sodium hydride (50% suspension in oil, 0.2 g, 1 equiv.) was added to a solution of *O,O*-diethyl thiophosphite (0.66 g, 1 equiv.) in diethyl ether (20 ml) and the mixture heated under reflux for 30 minutes. The reaction was cooled to 0° and

N-(benzyloxymethyl)-5-(chloromethyl)-tetrazole (1.2 g, 1 equiv.) was added dropwise with stirring which was continued for 48 hours. The ether was then removed *in vacuo* and the residue purified by silica chromatography (solvent A) to give diethyl *N*-(benzyloxymethyl)-5-thiophosphonomethyl)-tetrazole (1.3 g, 73%). ^{1}H n.m.r. ($CDCl_3$) δ 1.3 (6H t, J = 6 Hz) 3.75 (2H d, J = 18 Hz), 4.15 (4H m, J = 7 Hz), 4.6 (2H s), 5.9 (2 Hs), 7.3 p.p.m. (5H m, CIMS m/z 357 $[M + H]^+$.

This diethyl ester (1.3 g) was stirred for 84 hours in solution in trifluoroacetic acid at room temperature. Evaporation of excess TFA afforded diethyl 5-(thiophosphonomethyl)-1H-tetrazole (350 mg, 40%) after purification by silica chromatography (solvent C). ^{1}H n.m.r. ($CDCl_3$) δ 1.25 (6H t, J = 6 Hz), 3.77 (2H d, J = 18 Hz), 4.1 p.p.m. (4H m, J = 7 Hz), CIMS m/z 237 $[M + H]^+$, 254 $[M + NH_4]^+$.

Diethyl 5-(thiophosphonomethyl)-1H-tetrazole (150 mg, 1 equiv.) was heated in a sealed tube at 100° for 72 hours with iodotrimethylsilane (40 mg, 2 equiv.). The excess silane was removed *in vacuo* and the residue treated with 2 x 10 ml of 0.25 M Et_3NHHCO_3 (pH 8.7), the sample being lyophilised between each treatment. The product was purified by chromatography on a DEAE-Sephadex A-25 column (Et_3NH^+ form, 1.5 x 45 cm) with elution with a gradient of 0.25-0.75 M Et_3NHHCO_3 (pH 8.7) to give 5-(thiophosphonomethyl)-1H-tetrazole as the bis-triethylammonium salt (40 mg, 18%), ^{1}H n.m.r. (D_2O) 1.3 (18H t, J = 6.5 Hz), 3.5 (2H d, J = 18 Hz), 4.15 p.p.m (12H q, J = 7 Hz), Analysis C 38.52, H 9.31, N 21.5, $C_{14}H_{35}N_6O_2PS.H_2O$ requires C 40.53, H 9.31, N 21.0.

<u>*N*-Ethyl-5-(phosphonomethyl)-tetrazole (4)</u>.- 5-(Chloromethyl)-1H-tetrazole (1.2 g, 1 equiv.) was heated in triethyl phosphite (5.0 g, 3 equiv.) at 120° for 14 hours. Excess triethyl phosphite was then removed *in vacuo* and the residue purified by silica chromatography (solvent A) to give diethyl *N*-ethyl-5-(phosphonomethyl) tetrazole (1.9 g, 80%), ^{1}H n.m.r. ($CDCl_3$) δ 1.2 (6H t, J = 6 Hz), 1.5 (3H t, J = 6 Hz), 3.4 (2H d, J = 20 Hz), 4.1 (4H m, J = 7 Hz), 4.55 p.p.m. (2H q, J = 7 Hz), CIMS m/z 249 $[M + H]^+$, 266 $[M + NH_4]^+$.

This diethyl ester (1.0 g) was stirred for 2 hours at room temperature with bromotrimethylsilane (1.5 g). Evaporation of excess silane *in vacuo* gave a residue to which excess water was added. The latter was removed by repeated lyophilisation to leave a product which was purified by chromatography on a DEAE-Sephadex A25 column (Et_3NH^+

form, 1.5 x 45 cm) and elution with a gradient of 0-0.5 M Et_3NHHCO_3 (pH 8.4) to give the triethylammonium salt of *N*-ethyl-5-(phosphonomethyl)-tetrazole which was converted into the free acid using a Dowex 50 (H^+ form) column, yield (320 mg, 41%, ^1H n.m.r. (D_2O) δ 1.45 (3H t, J = 6 Hz), 3.4 (2H d, J = 20 Hz), 4.6 p.p.m. (2H q, J = 7 Hz), ^{31}P n.m.r. (D_2O) 18.8 p.p.m., Analysis C 23.6, H 5.02, N 26.7, P 14.79% $C_4H_9N_4O_3P.H_2O$ requires C 22.86, H 5.20, N 26.67, P 14.76%.

Nuclear Overhauser enhancement measurements were carried out at 400 MHz on a pyridine solution of (4). When either the signals due to the protons of the methylene group alpha to the phosphorus atom (3.4 p.p.m.) or the protons of the methylene group attached to nitrogen (4.6 p.p.m.) were irradiated for 1.5 s before accumulation of the ^1H n.m.r. spectrum, no enhancement of any signals could be detected. When the signal due to the protons of the N-ethyl group (3.45 p.p.m.) of 1-methyl 2-pyrroleacetonitrile was irradiated under similar conditions, significant enhancement of the signals at 4.0 ($-CH_2CN$) and 6.7 p.p.m. (ring proton alpha to nitrogen atom) occurred.

3-(Phosphonomethyl-1H-1,2,4-triazole (5).- 3-(Chloromethyl-1H-1,2,4-triazole (3.0 g, 1 equiv.) which had been prepared as described[13], was heated with triethyl phosphite (12.5 g, 3 equiv.) at 120° for 12 hours. Excess triethyl phosphite was removed *in vacuo* and the residue purified by silica chromatography (solvent C) to afford diethyl 3-(phosphonomethyl)-1H-1,2,4-triazole (2.1 g, 35%), ^1H n.m.r. ($CDCl_3$) δ 1.3 (6H t, J = 6 Hz), 3.55 (2H d, J = 20 Hz), 4.2 (4H m, J = 7 Hz), 8.3 p.p.m. (1H s), CIMS m/z 220 $(M + H)^+$. The diethyl ester (1.5 g) was heated under reflux in concentrated HCl (10 ml) for 12 hours, the excess HCl was then removed *in vacuo* and the residue purified by chromatography on DEAE cellulose (Et_3NH^+ form) with elution by a gradient of 0-0.5 M Et_3NHHCO_3 (pH 8.4) to give triethylammonium 3-(phosphonomethyl)-1H-1,2,4-triazole. Using a Dowex 50 (Na^+ form) column this was converted into the disodium salt (700 mg, 44%), ^1H n.m.r. (D_2O) δ 3.3 (2H d, J = 20 Hz), 8.3 p.p.m. (1H s), ^{31}P n.m.r. (D_2O) δ 17.4 p.p.m., Analysis C 15.37, H 4.2, N 19.6, P 13.5%. $C_3H_3N_3O_3PNa_2H_2O$ requires C 16.0, H 2.67, N 18.67, P 13.8%.

Adenosine-5' Ester of 5-(Phosphonomethyl)-1H-tetrazole (6).- To a solution of the anhydrous pyridinium salt of 5-(phosphonomethyl)-1H-tetra-

zole (150 mg, 1 equiv.) and 2',3'-O-isopropylidene adenosine (184 mg, 1 equiv.) in DMF (4 ml) was added a solution of dicyclohexyl carbodiimide (600 mg) in DMF (3 ml). The mixture was stirred at 20° for 96 hours, water (5 ml) was added and the stirring continued for a further 24 hours. The reaction mixture was then filtered and the filtrate evaporated to dryness *in vacuo*. The residue was purified by chromatography on a DEAE cellulose (Et_3NH^+ form, 3.5 x 40 cm) column with elution by a gradient of 0-0.5 M Et_3NHHCO_3 (pH 8.2). 2',3'-O-isopropylidene adenosine 5'-[5-(phosphonomethyl)-1H-tetrazole] was isolated as the triethylammonium salt (90 mg, 25%), ^1H n.m.r. (D_2O), δ 1.3 (18H t, J = 6.5 Hz), 1.6 (3H s), 1.8 (3H s), 3.25 (12H q, J = 7 Hz), 3.45 (2H d, J = 20 Hz), 4.15 (2H m), 4.7 (1H m), 5.15 (1H m), 5.3 (1H m), 6.3 (1H d, J = 4 Hz), 8.3 (1H s), 8.45 p.p.m. (1H s).

Removal of the isopropylidene group and isolation of the free acid of (6) was achieved by passing the triethylammonium salt down a Dowex 50 (H^+ form) column to give (52 mg, 18%) product, ^1H n.m.r. (D_2O), δ 3.5 (2H d), J = 2 Hz), 4.3 (2H m), 4.5 (1H m), 4.6 (1H m), 5.1 (1H m), 6.3 (1H d, J = 4 Hz), 8.5 (1H s), 8.6 p.p.m. (1H s), Analysis C 34.9, H 4.63, N 27.0, P 6.8%, $C_{12}H_{16}N_9O_6P.4H_2O$ requires C 32.0, H 5.7, N 27.8, P 6.2%.

Thymidine-5' Ester of 5-(Phosphonomethyl)-1H-tetrazole (7).- The 3'-O-acetyl thymidine-5' ester of 5-(phosphonomethyl)-1H-tetrazole was prepared in 50% yield as the triethylammonium salt from 3'-O-acetyl thymidine and (1) as described above. The acetyl group was removed by treating this salt (340 mg) with methanol (10 ml) which had been saturated with gaseous ammonia. The product of this reaction after removal of excess methanol was purified by chromatography on a DEAE cellulose column (Et_3NH^+ form 2.5 cm x 45 cm) with elution by a gradient of 0-0.5 M Et_3NHHCO_3 pH 8.4 and finally converted into the free acid (140 mg, 54%) with the aid of a Dowex 50 (H^+ form) column. ^1H n.m.r. (D_2O) 1.7 (3H s), 2.3 (2H m), 3.345 (2H d, J = 20 Hz), 4.1 (3H m), 4.5 (1H m), 6.25 (1H m), 7.5 p.p.m. (1H s), Analysis C 35.10, H 4.45, N 20.65, P 8.19%, $C_{12}H_{17}N_6O_7P.H_2O$ requires C 35.47, H 4.46, N 20.68, P 7.62%.

Preparation of Bis-Tetrazoles and Tetrazole Carboxylic Acids

5,5'-Methylenebis-1H-tetrazole (8).- To a solution of malononitrile (2.2 g,

0.03 mol) in dry THF (150 ml) was added dry sodium azide (20.0 g, 0.3 ml) with stirring followed by dry, powdered aluminium trichloride (9.0 g, 0.03 mol). The mixture was heated for 14 hours under reflux in a nitrogen atmosphere, then cooled and acidified with 5 M HCl. The solution was extracted with ethyl acetate (3 x 100 ml), the combined extracts dried (Na_2SO_4) and the solvent removed *in vacuo*. The residue was recrystallised from acetonitrile to give 5,5'-methylenebis-1H-tetrazole (2.3 g, 50%) m.p. 208°, lit.[14] 215°, ^1H n.m.r. (D_2O) 4.7 p.p.m. (s), Analysis C 23.84, H 2.33, N 73.79%, $C_3H_4N_8$ requires C 23.70, H 2.65, N 73.66%, CIMS m/z 153 $[M + H]^+$ 170 $[M + NH_4]^+$.

5,5'-Ethylenebis-1H-tetrazole (9).- This was prepared in an analogous manner from succinonitrile, m.p. 234°, lit.[15] 230°, ^1H n.m.r. (D_2O) 3.5 p.p.m. (s), Analysis C 28.90, H 3.22, N 64.1%, $C_4H_6N_8$ requires C 28.90, H 3.64, N 64.1%, CIMS m/z 167 $[M + H]^+$, 184 $[M + NH_4]^+$.

1H-Tetrazole-5-ethanoic Acid (10).- A solution in DMF (100 ml) of ethyl cyanoacetate (11.5 g, 0.1 mol), ammonium chloride (5.9 g, 0.12 mol) and dry sodium azide (7.15 g, 0.11 mol) was heated at 100° for 16 hours under nitrogen. Excess DMF was removed *in vacuo* the residue dissolved in water (50 ml) and then acidified to pH 2.0 with HCl. This mixture was cooled to 5° when crystals separated out which were filtered off and washed with ice water. Recrystallisation from propan-2-ol gave ethyl 1H-tetrazole-5-ethanoate (7.6 g, 50%), m.p. 128°, lit.[14] 128-130°.

The ethyl ester (500 mg) was heated under reflux in 3M NaOH solution (20 ml) for 4 hours. This solution was neutralised with 5M HCl and then evaporated to dryness *in vacuo*. The residue was extracted with hot acetonitrile (10 ml), filtered, and the acetonitrile evaporated to dryness. The 1H-tetrazole-5-ethanoic acid obtained was recrystallised from acetonitrile, yield 360 mg (93%) m.p. 170°, ^1H n.m.r. (D_2O) δ 4.1 p.p.m. (s), CIMS m/z 129 $[M + H]^+$, 146 $[M + NH_4]^+$, Analysis C 28.17, H 3.10, N 43.83%, $C_3H_4N_4O_2$ requires C 28.13, H 3.15, N 43.74%.

1H-Tetrazole-5-(3'-propionic Acid) (11).- 5-(2-Cyanoethyl)-1H-tetrazole, a by-product formed in the synthesis of (9), was purified by silica chromatography (solvent C), m.p. 140-142°, ^1H n.m.r. (d^6-DMSO) δ 3.55 (2H t, J = 6 Hz) 3.8 p.p.m. (2H t, J = 6 Hz), CIMS m/z 124 $[M + H]^+$.

The cyanoethyl tetrazole (50 mg) was heated under reflux in solution in 20% HCl/glacial acetic acid (2.5 ml, 1/1 v/v) for 12 hours.

excess acid was removed *in vacuo* and the residue crystallised from proapn-2-ol to give 1H-tetrazole-5-(3'-propionic acid) (36 mg, 62%) m.p. 218-220° (dec.), ^{1}H n.m.r. (D_2O) δ 2.8 (2H t, J = 6.5 Hz), 3.22 p.p.m. (2H t, J = 6.5 Hz), CIMS m/z 143 $(M + H)^+$, Analysis, C 33.8, H 4.20, N 38.91% $C_4H_6N_4O_2$ requires C 33.8, H 4.25, N 39.42%.

DISCUSSION

In our hands, the most effective route for the preparation of 5-(phosphonomethyl)-1H-tetrazole (1) was by Arbusov reaction between triethyl phosphite and an N-protected 5-(chloromethyl)-tetrazole. If this reaction is carried out without protection of the ring nitrogen, a further reaction occurs during the Arbusov reaction resulting in the formation of the diethyl ester of N-ethyl 5-(phosphonomethyl)-tetrazole. The N-ethyl bond cannot easily be broken and hence the 1H-tetrazole cannot be prepared by this method. The ethyl group is probably attached to N-2 of the tetrazole ring as no nuclear Overhauser enhancement of signals in the 400 MHz ^{1}H n.m.r. spectrum of (4) could be observed when the signals due to the protons of either the methylene group attached to phosphorus or the methylene group attached to nitrogen were irradiated before accumulation of the spectrum. On the other hand, a significant nuclear Overhauser enhancement of the signals due to the protons of both the methylene group attached to carbon and the proton alpha to the ring nitrogen atom in N-methyl 2-pyroleacetonitrile (12) ocurred when the signal due to the protons of the N-methyl group was irradiated before accumulation of the spectrum.

We find that the benzyloxymethyl group[16] is excellent for the protection of the ring nitrogen atom as it is easily attached to the tetrazole ring, is stable under the conditions of the Arbusov reaction and is readily removed by treatment with trifluoroacetic acid at room temperature. In contrast to the report by Yaouanc et al.[8], we found that the 1,3-dipolar addition of azide ion to diethyl phosphonoacetonitrile gave negligible yields of the diethyl ester of (1). Other tetrazole analogues used in our investigations were either made by an adaptation of our synthesis of (1) or were made by previously published procedures. It is of interest that the Arbusov reaction between triethyl phosphite and 3-(chloromethyl)-1,2,4-triazole did not result in alkylation of the ring to a significant extent and the

major product of this reaction was diethyl 3-(phosphonomethyl)-1,2,4-triazole. The reason for this difference in behaviour of the chloromethyl triazole and tetrazole is not clear but it is possible that the difference in nucleophilicity between the ring nitrogen atoms of the two ring systems may result in this different behaviour.

Contrary to the previous report[8], we did not find that compound (1) was an effective inhibitor of the replication of either HSV-1 or HSV-2 when these viruses were tested in tissue culture (Table 2). Compound (1) was a weak inhibitor of cell free DNA polymerases induced by either virus strain but the concentrations producing 50% inhibition of the enzyme activity for either polymerase were considerably greater than that for phosphonoacetic acid (Table 2). Compound (1) was a moderate inhibitor of the RNA transcriptase of influenza virus A, the concentration producing 50% inhibition of this enzyme was approximately the same as that for phosphonoacetic acid (Table 1).

Both phosphonoacetic acid and (1) have similar affinities for zinc ion under the conditions of the transcriptase assay (pK_d, 5.5 and 5.6 respectively). Alteration in the structure of (1) such as ring alkylation (4), insertion of an additional methylene group between the tetrazole ring and the phosphonic acid residue (2) or replacement of the phosphonic acid by a carboxylic acid residue (10) gave compounds which were poor chelators of zinc ion (pK_d, < 4) and which were devoid of antiviral activity. Replacement of the phosphoryl oxygen in (1) by sulphur gives 5-(thiophosphonomethyl)-1H-tetrazole (3) and we prepared this compound by a Michaelis Becker reaction between the anion of *O*-*O*-diethyl thiophosphite and N-protected (5-chloromethyl)-tetrazole. The thio-analogue (3) was a good chelator of zinc ions (pK_d, > 6) and was a more effective inhibitor of HSV-1 DNA polymerase and influenza RNA transcriptase than the parent compound 5-(phosphonomethyl)-1H-tetrazole. The only other compound which had appreciable activity as an inhibitor of the DNA polymerases of HSV-1 or HSV-2 and the RNA transcriptase of influenza virus was 3-(phosphonomethyl)-1,2,4-triazole (5) which was a moderate chelator of zinc ion (pK_d, 4.7) (Table 1).

In an attempt to improve the uptake of (1) into cells and in view of the reported antiviral activity of nucleoside esters of phosphonoacetic acid[17], we prepared the adenosine-5′ and thymidine-5′ esters of (1). Both of these nucleoside esters were weak inhibitors of HSV-1 and HSV-2

replication in Vero cells although neither compound showed appreciable inhibitory activity against the DNA polymerases induced by these viruses or against influenza virus RNA transcriptase.

Thus, despite the previous report[8], and despite the similarity in properties between the tetrazole and carboxylic acid moieties, we do not believe that 5-(phosphonomethyl)-1H-tetrazole (1) shows promise as a potential antiviral agent.

ACKNOWLEDGEMENT

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TABLE 1

Effect of Tetrazole Phosphonic Acids and Analogues on
RNA Transcriptase of Influenza Virus A and their Ability to Bind Zinc Ions

Compound	I_{50}^{*} (μM)	pK_d, (Zn^{2+})
$HO_2CCH_2PO_3H_2$	350	5.5
(1)	275	5.6
(2)	550	4.6
(3)	130	> 6
(4)	> 1000	< 4
(5)	580	4.7
(6)	> 1000	n.d.
(7)	> 1000	n.d.
(8)	> 1000	< 4
(9)	> 1000	< 4
(10)	> 1000	< 4
(11)	> 1000	< 4

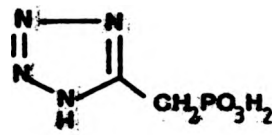
*Concentration producing 50% inhibition of enzyme activity.

TABLE 2

Effect of Tetrazoles on DNA Polymerases Induced by HSV-1 or HSV-2 and on Virus Replication in Vero Cells

Compound	DNA Polymerase I_{50} (μM)			HSV Plaque Reduction Assay I_{50} (mg/ml)	
	HSV-1	HSV-2	α	HSV-1	HSV-1
$H_2O_3PCH_2COOH$	10	10	40	0.022	0.025
(1)	220	275	> 1000	0.4	0.35
(2)	> 1000	> 1000	> 1000	> 1	> 1
(3)	90	n.d.	> 500	n.d.	n.d.
(4)	870	> 1000	> 1000	> 1	> 1
(5)	380	520	> 1000	0.7	0.82
(6)	850	900	> 1000	0.7	0.72
(7)	> 1000	> 1000	> 1000	0.85	0.9

Compounds (8-11) were inactive at ≤ 1000 μM (DNA polymerase assay or ≤ 1 mg/ml plaque reduction assay. No compounds were cytotoxic at concentration ≤ 1 mg/ml in the plaque reduction assay.



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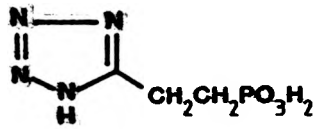

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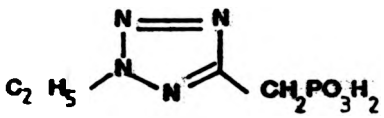

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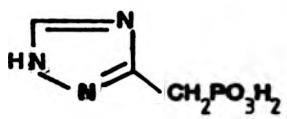

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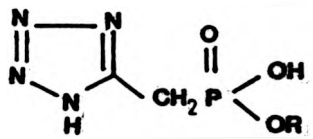
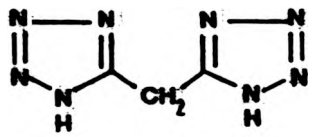


(6) R = adenosine – 5'

(7) R = deoxythymidine – 5'



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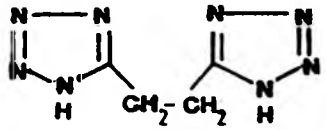

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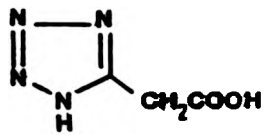

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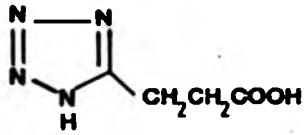

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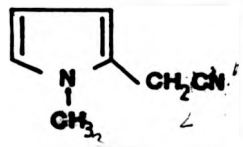

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TETRAZOLE PHOSPHONIC ACIDS AND ANALOGUES.

IRCS Medical Science: Biochemistry; Biomedical Technology; Cell and Membrane Biology; Drug Metabolism and Toxicology; Microbiology, Parasitology and Infectious Diseases; Pathology. [*IRCS Med. Sci.*, **13**, 1023-1024 (1985)]

The effect of pyrophosphate analogues on the replication of different strains of influenza virus A in tissue culture

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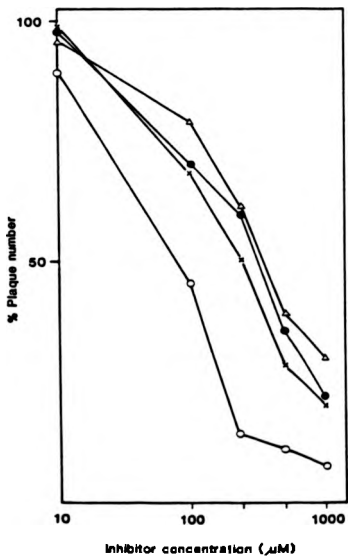
Pyrophosphate analogues such as phosphonoformate (foscarnet), thiopyrophosphates, and substituted methylene bisphosphonates, inhibit the replication of influenza virus A/X49 in tissue culture (1–3) by inhibiting the RNA transcriptase activity of this virus. Pyrophosphate analogues, in particular, phosphonoformate and phosphonoacetate (4), also inhibit the DNA polymerase induced by herpes viruses and it has been observed that different strains of herpes viruses exhibit different sensitivities to the inhibitors. For example, strains of HSV-1 and HSV-2 have been isolated which show complete resistance to phosphonoacetate or phosphonoformate and this resistance has been correlated with changes in the DNA polymerases induced by these viruses (5–7). No reports have been published so far on the sensitivities of different strains of influenza virus A to inhibition by pyrophosphate analogues, although it has been observed that the effect of rimantadine and ribavirin in combination on influenza virus replication in cell culture is dependent on the strain of virus used (8). We are studying (9) the effect of pyrophosphate analogues such as phosphonoformate and 5-phosphonomethyl-1H-tetrazole on the replication of influenza virus A in Madin-Darby canine kidney (MDCK) cells and wish to report our observations on the variation of inhibition with the virus strain used.

Materials and methods: A/X49[H3N2, Recombinant: A/England/864/75 X A/PR/8/34] was a gift from Dr N.J. Dimmock, Department of Biological Sciences, University of Warwick. A/X31 [H3N2, Recombinant A/PR/8/34 (H1N1) X A/Hong Kong/1/68 (H3N2)], A/R15 [A/R1/5*57(H2N2)], A/WS [A/WS/33 (H1N1)] were gifts from Dr K. Katrak, Roche Products Ltd, Welwyn, UK.



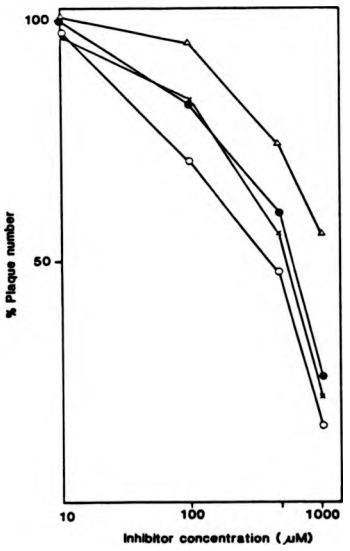


Figure 1 (left): Inhibition of plaque formation by strains of influenza virus A in MDCK cells by phosphonoformate. A/X49 (△), A/X31 (X) A/R15 (●) and A/WS (○). Figure 2 (right): Inhibition of plaque formation by strains of influenza virus A by 5-phosphonomethyl-1H-tetrazole A/X49 (△) A/X31 (X), A/R15 (●), and A/WS (○).

For the plaque reduction assay MDCK cells were seeded onto 5 cm plastic dishes at 3.0×10^6 cells/plate and the cells were grown at 37°C until confluent in maintenance medium. The confluent monolayers were washed with PBS and aspirated to dryness. Virus suspension (100 μl, 60 PFU) was added to the cell sheets and left for 15 min at room temperature. The test compound at the appropriate concentration (400 μl) was then added and left for 45 min before addition of the overlay which consisted of an agar medium containing 0.1% BSA, 2.5 μg/ml crystalline trypsin and 0.1% DEAE Dextran. The plates were incubated at 33°C for 4–6 days, stained with neutral red and the plaques counted.

Results and discussion: The results are presented in Figures 1 and 2. The sensitivities of our strains of influenza virus A to different concentrations of phosphonoformate (Figure 1) and 5-phosphonomethyl-1H-tetrazole (Figure 2) are shown. It may be seen that the A/WS strain was the most and A/X49 was the least sensitive. The reason for this difference in sensitivity is not known and we are studying the effect of pyrophosphate analogues on the RNA transcriptase activities of these viruses. However, we present these data at this time as we wish to draw to the attention of others that the strain of influenza virus A used may well affect results on the inhibition of virus replication by various compounds.

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INHIBITION OF VIRAL NUCLEIC ACID SYNTHESIS BY ANALOGUES OF INORGANIC PYROPHOSPHATE

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ABSTRACT

Analogues of inorganic pyrophosphate can inhibit the DNA polymerase of herpes simplex virus 1, or the RNA transcriptase activity of influenza virus A. Stuctural features of the pyrophosphate analogues which are essential for their inhibitory activity are discussed and these are related to the ability of the analogues to bind zinc ion.

INTRODUCTION

A number of nucleoside analogues such as acyclovir [1] and (E)-5-bromovinyl-2'-deoxyuridine (BVDU) [2] have recently been shown to possess antiviral activity and much effort has been spent recently on the synthesis of nucleoside analogues which might have better therapeutic properties than these two compounds. Both acyclovir and BVDU inhibit the synthesis of DNA in herpes-infected cells. Initially these two compounds are phosphorylated by virus-induced enzymes to give their 5'-monophosphates, these are then converted into the 5'-triphosphates which inhibit DNA synthesis in the virus-infected cells. Thus, the analogues must be metabolised before they can exhibit their antiviral properties and they are only active against a narrow range of viruses of the herpes family.

Compounds which inhibit nucleic acid synthesis in virus-infected cells without the necessity of a metabolic step might be expected to be active against a wider range of viruses than acyclovir or BVDU and might have clinical value so long as toxicity problems can be overcome. One such class of

inhibitors are analogues of inorganic pyrophosphate which can inhibit the reaction:

$$pppX \rightleftharpoons (pX)_n + pp_i$$

Phosphonoacetic acid (1) [3] was the first member of this family to be shown to possess antiviral activity and the related compound phosphonoformic acid (2) [4] is also an active antiviral agent. Both (1) and (2) inhibit DNA synthesis in cells which have been infected by herpes viruses and these compounds inhibit the DNA polymerases induced by these viruses.

$(HO)_2P(O)CH_2COOH$ (1) $(HO)_2P(O)COOH$ (2) $(HO)_2P(O)CH_2COOMe$ (3)

$MeO(HO)P(O)CH_2COOH$ (4) $(HO)_2P(O)CH_2CH_2COOH$ (5) $(HO)_2P(O)CR^1R^2P(O)(OH)_2$ (6)

The antiviral properties of a number of analogues of (1) and (2) have been examined and it has been observed that esterification of either the phosphoryl or the carboxyl groups to give for example (3) or (4) leads to a marked reduction in the antiviral activity [5]. Moreover, insertion of extra methylene groups between the phosphoryl and carboxyl groups as in (5) also reduces the antiviral activity. Enzyme kinetic [6] and other data [7] indicate that these compounds inhibit the herpes-induced DNA polymerases by acting directly on the enzymes without prior conversion into an active compound and that (1) and (2) probably act by chelating with an essential metal ion in the DNA polymerases induced by herpes viruses. Pyrophosphate analogues can also be effective inhibitors of the

RNA transcriptase activity of influenza virus A. Initiation of mRNA synthesis does take place in the presence of phosphonoformate (2) but elongation of the RNA chain does not occur [8].

We have studied a variety of pyrophosphate analogues and believe that their ability to inhibit nucleic acid synthesis in cells which have been infected with either herpes or influenza viruses is related to their ability to form stable chelates with a metal ion (probably zinc) at the active centres of the enzyme systems [9]. We have shown that there is a good relationship between the ability of the analogue to bind zinc ion at pH 8 and its effectiveness as an inhibitor of influenza mRNA synthesis [7]. We determine the binding constant of the analogue to zinc ion by a simple gel-filtration method at the pH optimum of the transcriptase reaction and from this derive the term pK_d', which we define as $-\log_{10}$ (dissociation constant of zinc ion - pyrophosphate analogue complex). The ionic species of the analogue which binds to the zinc ion is not defined in our term pK_d', as this merely refers to the ability of all ionic species present to bind zinc ion under given conditions, i.e., the conditions of the transcriptase assay. We find that if a pyrophosphate analogue has a $pK_d' > 6$ under these conditions then the compound is a good inhibitor of the transcriptase, on the other hand compounds with a $pK_d' < 5$ are in general poor inhibitors of the transcriptase (Table 1). The exact nature of the metal ion at the active centre of the transcriptase system has not been determined unequivocally. Addition of excess magnesium or manganese ion to the transcriptase assay system fails to affect RNA synthesis and

hence it is unlikely that these are the essential metal ions. It has been proposed [10] that most polymerase enzymes require zinc for activity and it has been shown the influenza viruses do contain zinc [11]. Addition of zinc ions to the transcriptase assay which has been partially inhibited by a pyrophosphate analogue restores the enzymic activity to a limited extent. However, zinc ion is toxic to the enzyme at high concentrations [12] and when excess zinc ion is added to the system full enzymic activity is not restored.

The results of our studies on pyrophosphate analogues can best be summarised by considering phosphonoacetic acid as the archetype pyrophosphate analogue and considering in turn the changes in biological activity which are brought about by varying the carboxyl, methylene and phosphoryl groups.

(a) Variation of the Carboxyl Group

As mentioned earlier, esterification of the carboxyl group in (1) leads to a loss of antiviral activity in cell free systems and a similar loss in activity is observed when the carboxyl group is replaced by an amide group. In animals, however, some carboxylic esters do have anti-herpes activty presumably because these esters are hydrolysed *in vivo* to liberate the free acid [5]. Replacement of the carboxyl group by a phosphoryl group gives rise to methylene bisphosphonic acid (6, R^1=R^2=H). While the parent compound is devoid of antiviral activity, as will be discussed later, replacement of hydrogen by an electron withdrawing group leads to compounds, e.g. (6, R^1=R^2=Cl) which are good inhibitors of the RNA transcriptase of influenza virus but which have little or no

anti-herpes activity.

Analogues of amino acids in which the carboxylic acid residue has been replaced by a 1H-tetrazole residue have pK_a values which are close to those of the parent amino acids and the tetrazole moiety has been used as an isostere for the carboxylic acid group in many pharmaceutically active compounds [13]. The recent report that 5-phosphonomethyl tetrazole (7) has anti-herpes activity [14] prompted us to synthesise a range of heterocyclic analogues of phosphonoacetic acid [15]. Treatment of 5-(chloromethyl)-1H-tetrazole with triethyl phosphite gave the diethyl ester of *N*-ethyl-5-(phosphonomethyl)-tetrazole presumably due to intramolecular attack by the tetrazole ring on the intermediate formed in the Arbusov reaction. The esterifying groups of the phosphonic acid residue could be removed by acid but *N*-ethyl-5-(phosphonomethyl)-tetrazole (10) was a very poor chelator of zinc ion and had no effect on the transcriptase of influenza virus. The ethyl group could not readily be removed from the tetrazole ring in this synthetic route, so we protected this ring with the benzyloxymethyl group before the Arbusov reaction. The diethyl groups and the benzyloxymethyl residue were readily removed from the Arbusov product with acid and 5-(phosphonomethyl)-1H-tetrazole (7) was obtained in high yield. The 5-(phosphonoethyl) analogue (9) was obtained in a similar fashion. It is interesting that the Arbusov reaction between 3-(chloromethyl)-1H-1,2,4-triazole proceeded smoothly without any intramolecular alkylation of the triazole ring. The bistetrazole (12) and the carboxylic acid (10) were prepared by standard methods.

We find that compound (7) and its triazole analogue (8) are weak inhibitors of the DNA polymerase induced by HSV-1. The 5-phosphonoethyl tetrazole (9) and the N-alkylated analogue (10) show no inhibitory activity against this enzyme. Of the four compounds, only (7) has significant inhibitory activity against the RNA transcriptase of influenza. The carboxylic acid (11) and the bis-tetrazole (12) do not inhibit either the DNA polymerase of HSV-1 or influenza RNA transcriptase. Only compound (7) binds zinc to an appreciable extent ($pK_{d'}$ = 5.6) and hence this parameter again is a pointer to the antiviral activity of these compounds (Table 2).

(b) Variation of the Methylene Group

The homologue of phosphonoacetic acid without a methylene group is phosphonoformic acid (2), a compound which is in general a more active antiviral agent than (1). The homologue of (1) with two methylene groups is 3-phosphonopropionic acid (3) a compound with litle antivral activity. The effectiveness of these compounds as inhibitors of influenza transcriptase is again reflected by their ability to bind zinc ion (Table 1). One reason for the gradation in $pK_{d'}$ values is that compound (2) forms a chelate with a five-membered ring, (1) forms a chelate with a six-membered ring and (3) forms a chelate with a seven-membered ring. It is well known that the order of stability of cyclic metal chelates is 5 > 6 > 7 [16]. Substitution of the methylene group with an alkyl or aryl group can lead to a loss of activity as a transcriptase inhibitor in cell-free systems. However, an increase in activity can be observed in tissue culture probably because these compounds are more lipophilic than the parent and

are taken up more readily by cells [17]. Replacement of the two hydrogen atoms of the methylene group in (1) by two chlorine atoms has a marked effect on the properties of the carboxyl group. The ability of dichlorophosphonoacetic acid to bind zinc ion is low (pK_d, < 4) and the compound does not inhibit the RNA transcriptase of influenza virus.

As mentioned above, methylene bisphosphonate (6, $R^1 = R^2 = H$) has little or no antiviral activity. Replacement of the bridge hydrogen atoms in (6) by electron withdrawing groups such as halogen increases the ability of these compounds to bind zinc ion and markedly increases their antiviral activity [7]. Replacement of the bridging methylene group by a carbonyl group (13) also leads to a good chelator of metal ions with both anti-herpes [18] and anti-influenza activity [7]. The presence of at least one electron withdrawing group on the bridging carbon atom in (6) and the observation that lipophilic pyrophosphate analogues are often more effective as inhibitors of viral replication in tissue culture than the polar parent compounds has led us to prepare [19] a number of C-alkyl monochloromethylene bisphosphonates. As may be seen in Table 3, these compounds are inhibitors of the RN transcriptase of influenza virus A in cell free systems and are inhibitors of viral replication in MDCK cells (Table 3). While the ability of these compounds to inhibit the transcriptase appears to be independent of chain length, the hexyl- and benzyl-substituted bisphosphonates are the most effective inhibitors of viral replication in cell culture. All are more effective than phosphonoacetic acid in both inhibitory systems.

(c) Variation in the Phosphoryl Group

Inorganic pyrophosphate at a sufficiently high concentration will inhibit RNA synthesis by the influenza transcriptase system (Table 1) [7]. Replacement of one or both phosphoryl groups by a thiophosphoryl group leads to mono-(14) and bis-(15)thiopyrophosphate. Both these compounds are considerably more effective than the parent pyrophosphate in inhibiting the replication of influenza virus A in MDCK cells and the synthesis of RNA by the transcriptase system [20]. Sulphur is a "soft" centre and oxygen is a "hard" centre on the Pearson "hard and soft" acid and base scale [9]. Both (14) and (15) are good chelators of zinc ion and it is possible that the increased antiviral activity shown by the thiopyrophosphates is related to the formation of stronger metal chelates in the transcriptase system of influenza virus than with pyrophosphate.

Removal of the oxygen between the phosphoryl groups of pyrophosphate gives rise to hypophosphate (16). While this has been shown to be an inhibitor of the DNA polymerase of herpes-infected cells [18], we have shown it to have little or no effect on the transcriptase of influenza virus. Similarly peroxydiphosphonate (17) is inactive against the transcriptase and the inactivity of (16) and (17) can be correlated with the abilities of these compounds to bind zinc ions (Table 2). In these latter two compounds, the change in distance between the two chelating phosphoryl groups may also be important.

Replacement of the phosphoryl group in (1) by the arsonate group to give arsonacetate (18) leads to a compound with anti-herpes activity [21] and with some anti-influenza

activity. Methylene bisarsonate (19) also shows little antiviral activity. Furthermore, toxicity problems associated with these arsenicals and with hypophosphate make them unattractive for clinical use.

$(HO)_2P(O)COP(O)(OH)_2$ (13)

$(HO)_2P(O)OP(S)(OH)_2$ (14)

$(HO)_2P(S)OP(S)(OH)_2$ (15)

$(HO)_2P(O)P(O)(OH)_2$ (16)

$(HO)_2P(O)OOP(O)(OH)_2$ (17)

$H_2O_3AsCH_2COOH$ (18)

$CH_2(AsO_3H_2)_2$ (19)

DISCUSSION

We believe that the evidence presented above strongly supports the sugestion that nucleic acid synthesis in cells which have been infected by either herpes or influenza viruses can be inhibited by pyrophosphate anlogues which act by forming a very stable chelate with an essential metal ion in the polymerase/transcriptase enzyme systems induced by the viruses. Before pyrophosphate analogues are adopted for clinical use, however, there are obvious toxicity problems to be overcome as many processes in the host cells involve the liberation of pyrophosphate and these may be affected by the presence of pyrophosphate analogues. One such problem is the accumulation of certain pyrophosphate analogues in bones and teeth [22]. This problem may be obviated by using these compounds topically to treat, for example, herpes infections. Another way in which this accumulation in bone might be overcome is to incorporate bulky groups into the analogues as this should make them less compatible with the hydroxy apatite

lattice of bones [23] and hence make them less likely to be incorporated into this lattice.

It is encouraging that many of the pyrophosphate analogues which inhibit virus-induced processes do not seem to be highly toxic to cells. For example, the thiopyrophosphates (14) and (15) inhibit the replication of influenza A virus in MDCK cells at concentrations which do not appear to affect the viability of the cells after 48 hours [19]. Furthermore, substituted methylene bisphosphonates inhibit the transcriptase of influenza virus A at concentrations at which they have little or no effect on the DNA polymerases of HSV-1 or -2, the DNA polymerase α [17] of mammalian cells (Table 1) and the DNA and RNA polymerases of *Escherichia coli* [7].

Recently, the effect has been studied of a large number of derivatives of phosphonoacetic acid on the DNA polymerase induced by HSV-2 and on the replication of HSV-2 in tissue culture [24]. The results of these investigations broadly agree with those outlined above except that it was observed that carboxylic esters of low molecular weight alcohols inhibited the DNA polymerase. This result is in contract with observations with HSV-1 [25] and our observations with the RNA transcriptase of influenza. The reason for this difference is not clear and deserves further investigation.

The hypothesis that pyrophosphate analogues inhibit virus replication by virtue of their chelating with an essential metal ion should lead to the design of compounds with significant biological activity but which incorporate features which reduce their toxicity or which lead to an increase in the uptake of the analogue into a particular region of the host.

EXPERIMENTAL

Materials

Arsonoacetate and methylene bisarsonate were gifts from Dr.H.B.F.Dixon, Cambridge University, U.K., hypophosphate was a gift from Dr. B. V. L. Potter, Leicester University, U.K., and peroxydiphosphonate was a gift from Professor N. J. Leonard, University of Illinois at Urbana-Champaign, U.S.A. C-Alkyl monochloromethylene bisphosphonates [19] and heterocyclic methylene monophosphonates [15] were prepared as described.

Biological Assays

The influenza virus strain used in these experiments was A/X49, a cross between A/England/864/75 and A/PR/8/34 with the H3N2 surface antigens of the A/England strain. RNA transcriptase assays and the determination of the antiviral activity of compounds in Madin Darby Canine Kidney (MDCK) cells were carried out as previously described [20]. The herpes simplex-1 virus strain used was KOS. The inhibition by pyrophosphate analogues of the DNA polymerase induced by this virus was carried by the method of Powell and Purifroy [26] and the DNA polymerase α assays were carried out by the method of Sabourin *et al.* [27]. The determination of pK_d, values related to the strength of the binding of zinc ion to the pyrophosphate analogues were determined by a gel-filtration method [7].

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TABLE 1

INHIBITORY EFFECT OF PHOSPHONOACETIC ACID ANALOGUES AND METHYLENE BISPHOSPHONATES OF THE RNA TRANSCRIPTASE OF INFLUENZA VIRUS A AND CALF THYMUS DNA POLYMERASE α [7]

Compound	pK_{d} (Zn^{2+})	Concn. (μM) Producing 50% Inhibition	
		Influenza RNA Transcriptase	DNA Polymerase α
RCH_2COOH	5.5	275	45
RCOOH	5.6	35	35
RCH_2CH_2COOH	< 4	> 500	> 500
RCHMeCOOH	~ 5	500	> 500
$RCONH_2$	< 4	> 500	> 500
$(EtO)_2P(O)CH_2COOH$	< 4	> 500	> 500
ROR	5.7	125	> 500
RNHR	5.7	50	> 500
RCH_2R	5.3	> 500	> 500
RCHClR	> 6	85	> 500
$RCCl_2R$	> 6	75	> 500
$RCBr_2R$	> 6	10	350
RCOR	5.4	20	100

Where R = $(HO)_2P(O)$

TABLE 2

INHIBITORY EFFECT OF PYROPHOSPHATE ANALOGUES AND HETEROCYCLIC METHYLENE PHOSPHONATES ON THE RNA TRANSCRIPTASE OF INFLUENZA VIRUS A AND THE DNA POLYMERASE OF HSV-1

Compound	pK_{d_2} (Zn^{2+})	Concn. (μM) Producing 50% Inhibition	
		Influenza A RNA transcriptase	HSV-1 DNA polymerase
Heterocyclic Analogues			
5-(Phosphonomethyl)-tetrazole(7)	5.6	275	220
3-(Phosphonomethyl)-1,2,4-triazole(8)	4.7	580	385
5-(Phosphonoethyl)-tetrazole(9)	4.6	550	> 1000
N-ethyl-5-(phosphonomethyl)-tetrazole (10)	< 4	> 1000	870
Tetrazole-5-ethanoic acid (11)	< 4	> 1000	> 1000
5,5'-Methylene bistetrazole(12)	< 4	> 1000	> 1000
Others			
Monothiopyrophosphate(14)	5.4	60	> 500
Bisthiopyrophosphate(15)	> 6	33	> 500
Hypophosphate(16)	5.1	> 500	150
Peroxydiphosphonate(17)	4.8	~ 500	n.d.
Arsonoacetate(18)	4.7	> 500	n.d.
Methylenebisarsenate(19)	~5	> 500	n.d.

n.d. not determined

TABLE 3

INHIBITORY EFFECT OF C-ALKYL MONOCHLOROMETHYLENE BISPHOSPHONATES $RCCl(PO_3H_2)_2$ ON THE RNA TRANSCRIPTASE OF INFLUENZA VIRUS A AND ON THE REPLICATION OF INFLUENZA VIRUS A IN MDCK CELLS

R	pK_d' (Zn^{2+})	I_{250}(%)	PR_{500}(%)
H	> 6	97	22
Me	n.d.	54	n.d.
Et	n.d.	61	n.d.
n-Pr	n.d.	55	34
n-Bu	> 6	64	37
n-Hex	> 6	59	83
CH_2Ph	> 6	58	60
PAA(1)	5.5	35	0

n.d. not determined

I_{250} = % inhibition of transcriptase at phosphonate concentration of 250 μM

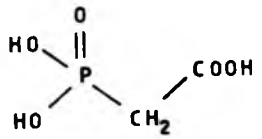
PR_{500} = % reduction of PFU (Virus Control = 7.7×10^8) caused by phosphate at a concentration of 500 μM

TABLE 4

EFFECTS OF PYROPHOSPHATE ANALOGUES ON EITHER THE HAEMAGGLUTININ TITRE OR THE VIRUS YIELD REDUCTION ASSAY OF INFLUENZA VIRUS A GROWN ON MDCK CELLS (MEAN OF TWO ASSAYS)

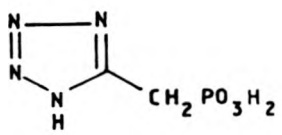
Compound	HAU ($\log_{10}$ U/ml)	PFU/ml
Cell control	< 2.0	≤ 10
Virus control	3.65	4.77×10^7
(14)	2.64	1.05×10^6
(15)	2.10	4.2×10^5

FIGURE 1 PHOSPHONOACETIC ACID



Phosphonoacetic Acid

FIGURE 2 HETEROCYCLIC ANALOGUES OF PHOSPHONOACETIC ACID



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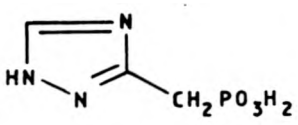

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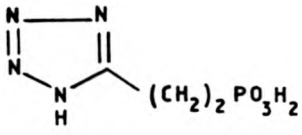

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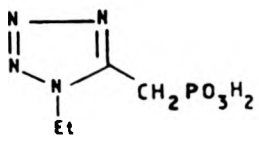

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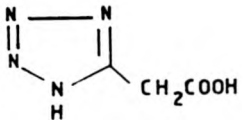

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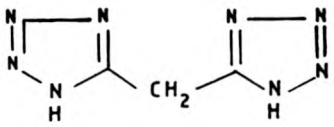
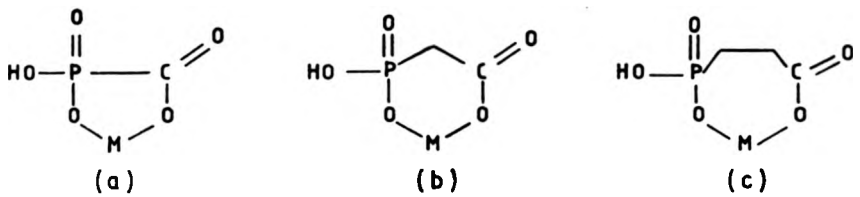


(12)

FIGURE 3 METAL CHELATES OF (A) PHOSPHONOFORMIC, (B) PHOSPHONOACETIC, AND (C) 3-PHOSPHONOPROPIONIC ACIDS.



Metal chelates of (a) phosphonoformic (b) phosphonoacetic and (c) 3-phosphonopropionic acids.

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Stress proteins are induced by hyperosmolarity in chick embryo fibroblasts

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A wide range of environmental stimuli including heat, oxygen deprivation, teratogens, heavy metals, ethyl alcohol, amino acid analogues etc., is known to induce the synthesis of a family of stress proteins in prokaryotic and eukaryotic cells (Ashburner & Bonner, 1979). The functions of these inducible stress proteins are poorly understood. Schlesinger *et al.* (1982) have hypothesized that the induction of these proteins could defend the cell exposed to an environmental insult from further damage by modifying the nuclear and the cytoplasmic cytoskeleton so to protect the metabolic apparatus from the stimulus persistance and to adapt consequently. In addition, the process of cell adaptation to adverse environmental factors is known to involve the cell's ability to modify the chemical complexion of the membrane and to alter the activity of membrane-associated enzymes including transport processes (Quinn, 1983).

We have recently shown that the exposure of chick embryo fibroblasts to a hyperosmolar medium results in an increase of amino acid-transport activity. This change appears to be restricted to the A system of mediation for neutral amino acids, to be dependent on both active transcription and translation, and to require a continuous exposure of the cells to the hyperosmolar stimulus (Tramacere *et al.*, 1984*a*,*b*).

In pursuing a correlation between the probable increase in the number of transport molecules and the alteration of gene expression in cells exposed to a hyperosmolar environment, we studied the pattern of polypeptides synthesized during the adaptation process to a hyperosmolar stress.

Secondary cultures of chick embryo fibroblasts were exposed to 0.5 osM in complete culture medium. Labelling was performed between 0.5 and 3 or 18.5 and 21 h of the hyperosmolar treatment. Cells were then solubilized in SDS/sample buffer and the labelled proteins were separated on 10% (w/v) SDS/polyacrylamide slab gel (Laemmli, 1970) and detected by fluorography as described by Bonner & Laskey (1974).

The effect of cell exposure for 3 or 21 h to a 0.5 osM medium on the pattern of protein synthesis was examined. When the pattern of polypeptides synthesized during the treatment was compared with those synthesized by untreated cells, the synthesis of certain proteins appeared reduced whereas for others there was a clear enhancement. As shown in Fig. 1, after 3 h of hyperosmolar treatment four proteins with apparent M_r of 76 000, 59 000, 51 000 and 38 000 were synthesized in smaller amounts than in control cells, meanwhile others (M_r 63 000 and 36 000) were synthesized in greater amounts. When cells were labelled between 18.5 and 21 h of hyperosmolar treatment, the synthesis of those polypeptides that at 3 h of hyperosmolar exposure appeared reduced or increased, returned to control values. Moreover, the synthesis of M_r 57 000 polypeptide was markedly increased. These preliminary data suggest that the expression of some specific proteins changes at specific times during the cell adaptation to hyperosmolarity. At early times there is a change in the

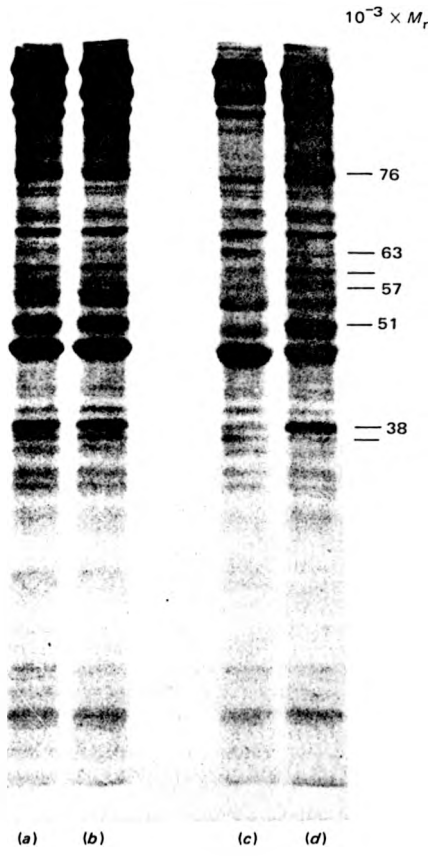

Fig. 1. *Pattern of proteins synthesized in hyperosmolarity-treated chick embryo fibroblasts*

Cells were exposed to 0.5 osM in complete culture medium. The labelling procedure was performed with 10 μCi of [^{14}C]glycine/ml between 0.5 and 3 or 18.5 and 21 h of the hyperosmolar treatment. Shown is a fluorograph of the gel. Lanes (*a*) and (*b*): controls at 3 or 21 h of incubation, respectively. Lanes (*c*) and (*d*): cells exposed to 0.5 osM for 3 or 21 h, respectively.

Abbreviation used: SDS, sodium dodecyl sulphate.

synthesis of several polypeptides, whereas at later times the protein pattern appears to be similar to control.

Our unpublished observations on the behaviour of such parameters as polyribosome profile, cation content and amino acid transport have shown a strong perturbation of these parameters in the first few hours of hyperosmolar treatment followed by a slow but persistent return to the values of the control. These results are an indication of the capacity of the cells to cope with an osmotically altered environment and are compatible with the above described modulation of protein synthesis. Of course, the possibility that the change in the expression of specific proteins might be referred to the alteration of some functions in hyperosmolarity-treated cells deserves further study.

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Pyrophosphate analogues as inhibitors of viral polymerases

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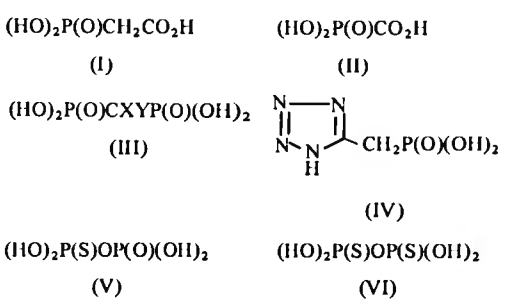
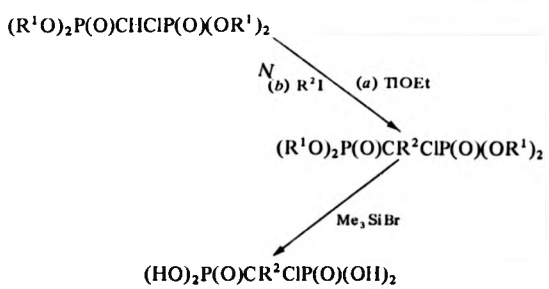
Analogues of pyrophosphoric acid, e.g. phosphonoacetic (I), phosphonoformic (II) and *C*-substituted methylene bisphosphonic acids (III) and the related tetrazole (IV) can act as product inhibitors of viral polymerases. For example, the replication of herpes viruses can be inhibited by (I) (Boezi, 1979) or (II) (Oberg, 1983), both compounds inhibiting the DNA polymerases induced by these viruses. In addition, (I), (II), (III, X = Y = Cl) and (IV) can inhibit the RNA transcriptase activity of influenza virus A (Cload & Hutchinson, 1983; Stridh & Datema, 1984).



The inhibition of the RNA transcriptase activity of influenza virus by these compounds appears to be related to their abilities to form complexes with an essential metal ion (probably zinc) in the transcriptase (Cload & Hutchinson, 1983; Hutchinson 1985). Thus (III, X = Y = H) forms only weak complexes with zinc ions at physiological pH while (III, X = Y = Cl) forms considerably stronger complexes. This behaviour is paralleled in their inhibitory activities and (III, X = Y = Cl) is a good inhibitor of the RNA transcriptase activity while (III, X = Y = H) is a poor inhibitor.

The metal chelating properties of inorganic pyrophosphate are affected if oxygen atoms are replaced by sulphur. Oxygen is a 'hard' centre of the Pearson Hard and Soft Acid and Base Scale while sulphur is a 'soft' centre (Pearson, 1968). Thus, thiopyrophosphates might be expected to bind to Zn^{2+} ions in a manner different to that observed with pyrophosphate. We have shown by ^{31}P n.m.r. that bisthiopyrophosphate (VI) appears to bind to Zn^{2+} through sulphur while it binds to Mg^{2+} through oxygen (Hutchinson *et al.*, 1985). Mono- (V) and bis-thiopyrophosphate (VI) form strong complexes with Zn^{2+} ions under physiological conditions. They are as good inhibitors of the RNA transcriptase activity of influenza virus A as compounds (I)–(IV) and are better inhibitors of the transcriptase than inorganic pyrophosphate. Mono-and bis-thiopyrophosphate inhibit the replication of influenza virus A in MDCK cells and do not appear to be cytotoxic to these cells after 3 days at concentrations which cause marked inhibition of virus replication.

Lipid-soluble pyrophosphate analogues should be taken up by cells more readily than highly polar compounds, e.g. (I)–(III). The presence of an electron-withdrawing group on the bridge carbon atom of methylene bisphosphonates is necessary if these bisphosphonates are to be good inhibitors of the RNA transcriptase of influenza virus A (Cload & Hutchinson, 1983). We have developed a method for the synthesis of *C*-alkylated monochlorobisphosphonates (VII) in high yield:



The key steps in our synthetic route are the use of thallium(I) ethoxide as base to form the anion from a tetra-alkyl ester of monochloromethylene bisphosphonate followed by reaction with an alkyl iodide and de-esterification with trimethylsilyl bromide (Hutchinson & Semple, 1985). In this way (VII) can be prepared in over 80% yield overall. If the sodio- or lithio-salts of the monoanion of the tetra ester of monochloromethylene bisphosphonate are used in the alkylation step or if de-esterification is carried out by hydrolysis with concentrated acid, marked reductions in the yield of (VII) occur. In preliminary experiments, we have shown that (VII, R = Me, n-Pr, n-Bu, n-Hex) are effective inhibitors of

the RNA transcriptase activity of influenza virus A as this activity is inhibited by more than 50% when the analogues are present at a concentration of 250 μM.

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The major P^1,P^4-bis-(5′-adenosyl)-tetraphosphate-binding protein in *Artemia* is a protein kinase

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Several acid-soluble nucleotides act as specific signals in the regulation of metabolic reactions (Tomkins, 1975). The past decade has seen a considerable growth in interest in the novel purine nucleotide P^1,P^4-bis-(5′-adenosyl)-tetraphosphate (Ap_4A). This apparently ubiquitous component of living cells has been shown to alter its intracellular concentration up to 1000-fold during the G_1 to S progression of a synchronized cell cycle (Weinmann-Dorsch *et al.*, 1984). It may be involved in a number of metabolic processes in the cell including the initiation of DNA replication (Weinmann-Dorsch *et al.*, 1984) and stimulation of processing of ADP-ribosylated proteins (Surowy & Berger, 1983). It is synthesized by the aminoacyl-tRNA synthetases in the presence of Zn^{2+} ions (Goerlich *et al.*, 1982). Evidence to date suggests a role as a messenger molecule in cellular proliferation.

As part of our investigations into the function of Ap_4A we have previously examined the levels of this nucleotide during development of the brine shrimp *Artemia*. When ametabolic encysted gastrulae of *Artemia* resume development they do so in the total absence of DNA replication and cell division until the point of hatching (16 h) although protein and RNA synthesis proceed unhindered. Upon reinitiation of development, a 125-fold rise in the intracellular concentration of Ap_4A was observed, the maximum level coinciding with hatching and the onset of DNA synthesis (McLennan & Prescott, 1984).

In order to clarify the relationship between Ap_4A, DNA synthesis and cellular growth and division we have sought to identify the intracellular target proteins of this nucleotide. We report here that the major protein isolated from newly hatched larvae which is capable of binding Ap_4A is a protein kinase.

When extracts of larvae are analysed by sucrose density gradient sedimentation analysis and the binding of [^{3}H]Ap_4A determined in each fraction by a nitrocellulose filter binding assay, a major binding protein of M_r 93 000 (4.8S) is observed which co-sediments with an enzymic activity capable of incorporating [^{32}P]orthophosphate into histone H2B using [γ-^{32}P]ATP as substrate.

By contrast, no evidence has been obtained to suggest that this binding protein may be associated with the DNA polymerase–α-holoenzyme complex in this organism, as has been reported previously for Ap_4A-binding proteins from calf thymus (Grummt, 1978) and HeLa cells (Rapaport *et al.*, 1981).

The *Artemia* binding protein has been partially purified from newly hatched larvae by ammonium sulphate precipitation, Matrex Blue and DEAE-cellulose chromatography and AcA 34 gel filtration. This scheme was designed to remove phosphodiesterases and all other enzyme activities capable of degrading the ligand, thus allowing a more detailed study of the protein. The purified Ap_4A-binding protein still possesses protein kinase activity.

Using histone H2B (the preferred substrate) and [γ-^{32}P]ATP of medium specific activity (1.4 Ci/mmol), stimulation of the kinase activity was observed in the presence of 1 μM-cyclic AMP and 1 mM-cyclic GMP (2.1 and 2.0-fold respectively). However, at similar concentrations, Ap_4A had no detectable effect on kinase activity. Calmodulin and Ca^{2+} were also without effect.

The preparation was found to possess endogenous acceptor polypeptides when [γ-^{32}P]ATP of high specific activity was used (> 5000 Ci/mmol). After separation on a 10% (w/v) polyacrylamide gel, four major acceptor species of M_r 35 000, 40 000, 42 000 and 72 000 were observed. These polypeptides all appear to be part of a single protein complex, as shown by their co-sedimentation on a 5–20% sucrose gradient. Since most protein kinases undergo autophosphorylation *in vitro* when incubated with [γ-^{32}P]ATP of high specific activity (de Jonge & Rosen, 1977), such a mechanism may be responsible for the incorporation observed here. The precise subunit composition of the native kinase is at present unclear, but our observations may be the result of partial proteolysis of a larger precursor. Such an observation has been made by several investigators (Kuo & Shoji, 1982).

The polypeptides of M_r 35 000 and 40 000 or 42 000 may represent the proteolytic products of the M_r 72 000 polypeptide which, when associated with the third polypeptide of low molecular weight, yields the native kinase of M_r 93 000.

This interpretation is supported by the fact that autophosphorylation of crude extracts in the presence of sodium fluoride, an ATPase and protein phosphatase inhibitor, yields only the polypeptide of M_r 72 000 on sodium dodecyl sulphate/polyacrylamide gel. This polypeptide co-sediments with the native Ap_4A-binding protein of M_r 93 000 on sucrose gradients.

Cyclic AMP (1 μM) or cyclic GMP (1 μM) completely suppress the phosphorylation of the bands of M_r 42 000 and 40 000 while cyclic GMP also reduces phosphorylation of the polypeptides of M_r 72 000 and 35 000. The possible role of this kinase in growth regulation and development in *Artemia* and the significance of Ap_4A binding are currently under investigation.

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The role of an upstream sequence in the transcription of a human transfer RNA gene

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A 2.4 kilobase *Hind*III fragment of human genome DNA which hybridized tRNA was isolated from a larger fragment cloned in λ Charon 4A and subcloned into the plasmid pAT153. This recombinant was found to be of remarkably high transcriptional activity when microinjected into *Xenopus* oocyte nuclei, producing more tRNA than a comparable cloned cluster of seven active *Xenopus* tRNA genes (Clarkson *et al.*, 1978). Southern blot analysis (Southern, 1975) of restriction fragments and determination of the sequence (Sanger *et al.*, 1980) of 1250 residues within the 2.4 kilobase fragment revealed the presence of a single $tRNA^{Glu}$ gene (residues 356–427 inclusive of the sequenced fragment) in the middle of the 2.4 kilobase fragment. Immediately preceding this gene there is a sequence which can be folded into a tRNA-like structure (Goddard *et al.*, 1983).

The separation of the intragenic promoter elements of eukaryotic tRNA genes is variable. This variability would be eliminated, to allow common recognition and binding of RNA polymerase III and/or transcription factors, if the gene itself adopted a higher-order tRNA-like structure (Hall *et al.*, 1982). These observations, together with the finding that the 5′-flanking, or upstream, sequence of some tRNA genes inhibits transcription (see, e.g., Dingermann *et al.*, 1982; Hipskind & Clarkson, 1983), led us to ask whether the potential for a tRNA-like structure within the upstream sequence of this very active human $tRNA^{Glu}$ gene serves to enhance transcription of the gene. We therefore constructed a series of recombinants in which parts of the upstream sequence were deleted and present below our initial results and tentative conclusions on the transcriptional activity of some of these.

The $tRNA^{Glu}$ gene, cloned as a 937 base-pair fragment in Ml3mp9 for sequence analyses, was subcloned further as a 489 base-pair *Eco*RI–*Nae*I fragment in the same vector. There were no suitably positioned restriction sites to allow simple deletion of only the tRNA-like upstream sequence (residues 285–355). However, a unique *Sst*I site (297–302) in the recombinant allowed access to this region for sequential removal of nucleotides by partial digestion with *Bal*31 exonuclease. Subsequent cleavage by *Stu*I at another unique site after nucleotide 151 upstream from the *Sst*I site, resulted in deletions which contained a common sequence (residues 1–151) of human genomic DNA before the deletion. With no hydrolysis by *Stu*I, deletions were confined largely to the tRNA-like flanking sequence.

The extent of deletion in each of the generated mutants was estimated from the size of the fragments released on digestion of the DNA with *Sma*I (⩽259 base-pairs or ⩽404 base-pairs in the absence of digestion by *Stu*I). A suitable range of deletions were selected and characterized by sequence analysis. Supercoiled replicative form RNA, free of contaminating host DNA, was prepared from these recombinants and quantified spectrophorometrically.

DNA (10 ng per oocyte) was co-injected with [α-^{32}P]-GTP (410 Ci/mmol; 0.5 μCi per oocyte) into *Xenopus* oocyte nuclei (10 oocytes per recombinant) and incubated overnight (Bienz & Gurdon, 1982). Total RNA was extracted from the ten pooled oocytes and separated on 10% polyacrylamide gels containing 4 M-urea (Kressman *et al.*, 1978). The newly synthesized RNA was detected by autoradiography of the gel. The amounts of RNA transcribed from three different recombinants was quantified by measurements of the radioactivity of the tRNA and pre-tRNA in the gel bands. These recombinants were Glu6, containing the intact sequence, D5 from which residues 152–305 inclusive had been deleted to remove the first arm of the potential tRNA-like upstream sequence, and D7 where the potential for the first two arms of that structure had been destroyed by removal of residues 152–311 inclusive. The decrease in RNA produced from 100% (Glu6) to 42% (D5) to 19% (D7) indicates that deletion of increasingly large portions of the tRNA-like structure in the upstream sequence of this human $tRNA^{Glu}$ gene gradually decreases the transcriptional activity of that gene. Since these preliminary results support the hypothesis that transcription of the gene is enhanced by this unique upstream sequence, we are pursuing further studies on the transcriptional activity of a wider range of the characterized deletion mutants.

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Thio-analogues of inorganic pyrophosphate inhibit the replication of influenza virus A in vitro

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Summary

Mono- and bisthiopyrophosphate can inhibit the replication of influenza virus A/X49 in Madin–Darby canine kidney (MDCK) cells at concentrations at which no cytotoxic effect is observed after 3 days. The thiopyrophosphate analogues inhibit the RNA transcriptase activity of this virus possibly by chelating with an essential metal ion in the transcriptase complex. [^{31}P]NMR spectroscopy indicates that bisthiopyrophosphate coordinates to zinc through sulphur and magnesium through oxygen which may influence the inhibitory properties of this compound with metal-containing enzymes.

influenza; inhibition; monothiopyrophosphate; bisthiopyrophosphate; transcriptase

Introduction

The antiviral activity of pyrophosphate analogues such as phosphonoacetic (1) [1], phosphonoformic (2) [2] and substituted methylene bisphosphonic (3) [3] acids is well known. We have recently observed that the inhibitory activity of these compounds on the RNA transcriptase activity of influenza virus A/X49 appears to be related to their metal-chelating properties, and, in particular, to their ability to complex with zinc ions. On the Pearson 'hard and soft' acid and base scale, zinc ions are classified as 'intermediate' and hence should form complexes with both hard and soft ligands [4]. We now report on the effect of thiopyrophosphates (4) and (5) which contain a potential soft ligand (sulphur) on the replication of influenza virus A/X49 and on its RNA transcriptase.

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$(HO)_2P(O)CH_2COOH$ (1)

$(HO)_2P(O)COOH$ (2)

$(HO)_2P(O)CCl_2P(O)(OH)_2$ (3)

$(HO)_2P(S)OP(O)(OH)_2$ (4)

$(HO)_2P(S)OP(S)(OH)_2$ (5)

Materials and Methods

Monothiopyrophosphate (4) and bisthiopyrophosphate (5) were prepared as described previously [5,6] and the determination of zinc ion stability constants (K_d) were carried out as described [3].

Virus type and preparation

The influenza virus A/X49 was a cross between A/England/864/75 and A/PR/8/34 with the H3N2 surface antigens of the A/England strain. The virus was grown in the allantoic sac of fertile hens' eggs and was isolated essentially as described [7]. Eleven-day-old embryonated hens' eggs were inoculated with infected allantoic fluid (0.1 ml of a 10^{-3} dilution in phosphate-buffered saline (PBS)), the eggs were incubated (33°C/48 h) and then chilled (–20°C/2 h). The allantoic fluid was collected and centrifuged (3000 r.p.m./20 min) to remove unwanted egg membranes; from this point onwards all procedures were carried out at 0–4°C. The supernatant was removed and the virus was pelleted by centrifugation (21 000 r.p.m./90 min, 6 × 250 ml rotor). The supernatant was discarded and the virus pellet was allowed to soak overnight in PBS. The pellet was then resuspended in PBS and layered onto a velocity gradient of 10–40% (w/v) sucrose in buffer (30 ml, 10 mM Tris-HCl, pH 7.4) and centrifuged (22 000 r.p.m./1 h, 3 × 65 ml swing-out rotor). The diffuse virus band was collected by bottom puncture and the sucrose was diluted out with PBS to a final volume of 30 ml. The virus suspension was layered onto an equilibrium gradient of 30–70% (w/v) sucrose in buffer (30 ml, 10 mM Tris-HCl, pH 7.4) and centrifuged (20 000 r.p.m./overnight, 3 × 65 ml swing-out rotor). The virus band was collected, diluted with PBS and the virus pelleted by centrifugation (30 000 r.p.m./2 h, 8 × 50 ml rotor). The supernatant was discarded and the pellet was allowed to soak overnight in PBS. The virus was then resuspended in buffer (3 ml, 400 µM Tris-HCl, pH 8.0) and frozen as aliquots at –70°C and thawed once prior to use.

RNA transcriptase assay

The standard reaction mixture contained in 200 µl: 50 mM Tris-HCl, pH 8.0; 5 mM magnesium acetate; 150 mM potassium chloride; 5 mM dithiothreitol; 0.4 mM ApG; 0.25% (v/v) Nonidet P-40; 0.4 mM each of ATP, CTP, GTP and [^{3}H]UTP (5 µCi) and purified virus (10 µl, 2000 HAU). Mixtures were kept at 4°C until zero time of

reaction, polymerisation being initiated by addition of virus. The mixture was kept at 30°C for 12 h during which time the incorporation of tritium into acid-precipitable material increased in a linear fashion. After 1 h, cold saturated sodium pyrophosphate solution (200 µl) followed by cold TCA (2 ml, 10% w/v) was added and the mixture kept on ice for 15 min after thorough agitation. Precipitated material was collected on Whatman GF/C discs which were washed several times with 10% TCA, once with ethanol and dried. The radioactivity of material precipitated on the discs was then determined by scintillation counting using a toluene-based medium. Pyrophosphate analogues were added to the reaction mixtures before addition of virus. The concentration of analogue which inhibited by 50% the incorporation of [^{3}H]uridine into acid-insoluble material was derived from the dose–response curve for each compound.

Antiviral activity in vitro

The haemagglutinin titre of the virus grown at low multiplicity on MDCK cells was determined with and without added pyrophosphate analogue in the following manner. Glass vials (1 cm diam.) were seeded with 2×10^5 MDCK cells and 1 ml of maintenance medium (Dulbecco's modified Eagle's medium; DMEM) containing glutamine (200 µM), 5% foetal calf serum and penicillin/streptomycin (50 units/ml) was added. The cells were incubated overnight at 37°C in a CO_2 incubator, then the maintenance medium was removed and the cell sheet washed twice with a solution of 0.1% BSA in PBS. The vials were inoculated with influenza virus A/X49 at a m.o.i. of 0.01 infectious virus particles/cell in 100 µl volume. After the virus had been allowed to absorb onto the cells for 1 h at room temperature, the inoculum was removed and 500 µl of maintenance medium (DMEM + glutamine + 0.1% BSA + penicillin/streptomycin + 25 µg trypsin/ml) which contained 0, 10, 50 or 100 µM of test compound were added. The cells were incubated at 33°C for 3 days in a gassed incubator after which time the medium was removed and the haemagglutinin assay carried out (Table 1).

A plaque assay was also carried out in the following manner. MDCK cells were seeded onto 5 cm plastic dishes at 3.0×10^6 cells/plate. These were grown at 37°C until confluent in maintenance medium. The confluent monolayers were washed with PBS and aspirated to dryness. Virus suspension (100 µl) was added to the cell sheets and left for 1 h at room temperature. The inoculum was removed and the cells were overlaid with an agar medium which contained 0.1% BSA, 2.5 µg/ml crystalline trypsin and 0.1% DEAE Dextran. The plates were incubated at 33°C for 4–6 days, stained with neutral red and the plaques counted. The results are presented in Table 2. Samples from the last wash of the cell monolayers after absorption of the virus were retained and plaques to show that haemagglutinin and infectious virus yields were not due to residual non-absorbed virus.

No cytotoxic effects could be observed with either thiopyrophosphate at 100 µM after 3 days.

TABLE 1

Effect of mono- and bisthiopyrophosphates on haemagglutinin titre of influenza virus A/X49 grown on MDCK cells (mean of two assays)

Compound	Concentration (μM)	HAU (log_{10} units/ml)
Cell control		<2.0
Virus control	0	3.65
Monothiopyrophosphate	100	2.64
	50	3.10
	10	3.45
Bisthiopyrophosphate	100	2.10
	50	2.70
	10	3.60

TABLE 2

Yield reduction assay by measurement of infectious influenza A/X49 virus grown on MDCK cells (mean of two assays)

Compound	Concentration (μM)	PFU/ml
Cell control		$\leqslant 10$
Virus control	0	4.77×10^7
Monothiopyrophosphate	100	1.05×10^6
	50	1.5×10^7
	10	4.25×10^7
Bisthiopyrophosphate	100	4.2×10^5
	50	9.7×10^6
	10	5.0×10^7

[^{31}P]NMR measurements

These were carried out at 308 K and 36.44 MHz on a Bruker WH90 spectrometer, chemical shifts are recorded relative to H_3PO_4 (0 ppm). Solutions of tetrasodium pyrophosphate or tetra(triethylammonium)bisthiopyrophosphate (15 mg/ml) were prepared in 0.1 M triethanolamine hydrochloride buffer, pH 8.2, the latter being made up in water which had previously been passed down a small Chelex 100 chelating column (Na^+ form, Biorad Inc.). To the pyrophosphate or bisthiopyrophosphate solution (1 ml, 1 mol) was added a solution in deuterium oxide (also treated with Chelex 100, 1 ml) which contained 0.8 mol of magnesium chloride, zinc chloride or cadmium bromide. After mixing the [^{31}P]NMR spectra were recorded (Table 3).

Results

Both mono- and bisthiopyrophosphate inhibit the replication of influenza virus

TABLE 3

[^{31}P]NMR chemical shifts (ppm) of solutions of pyrophosphate or bisthiopyrophosphate at pH 8.2 after the addition of metal ions

Metal	P_2O_7	$P_2O_4S_2$
None	-6.045	29.693
Mg^{2+}	-5.254	32.418
Zn^{2+}	-4.839	21.849
Cd^{2+}	-5.643	20.714

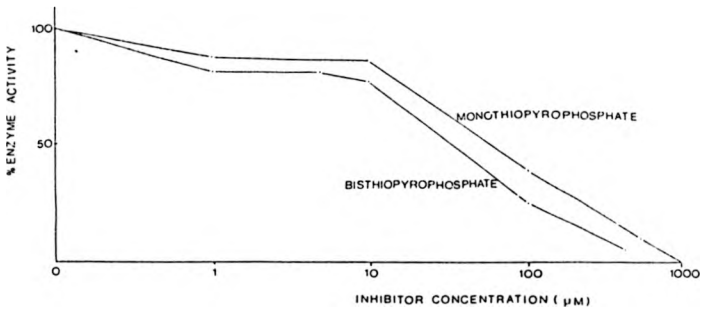

Fig. 1. Inhibition of RNA transcriptase of influenza virus A/X49 by mono- and bisthiopyrophosphate.

A/X49 in MDCK cells (Table 1). The haemagglutinin titre of 3.65 $\log_{10}$ units/ml for the control in the absence of thiopyrophosphate was reduced to 2.64 $\log_{10}$ units/ml for mono- and 2.10 $\log_{10}$ units/ml for bisthiopyrophosphate at 100 μM. Similarly a virus yield of 4.77×10^7 PFU/ml in the virus control was reduced to 1.05×10^6 PFU/ml for mono- and 4.2×10^5 PFU/ml for bisthiopyrophosphate at 100 μM. No cytotoxic effect was observed with either compound after 3 days in our assays. The compounds inhibit the RNA transcriptase activity of the virus (Fig. 1) producing a 50% inhibition of the transcriptase at 60 μM (monothiopyrophosphate) and 33 μM (bisthiopyrophosphate). Both compounds were good chelators of zinc ions and the $pK_{d'}$ at pH 8.2 [3] was 5.4 (monothiopyrophosphate) and 6.3 (bisthiopyrophosphate); under similar conditions inorganic pyrophosphate had a $pK_{d'}$ of 5.7. From the [^{31}P]NMR spectra of solutions of bisthiopyrophosphate after additions of metal ions it appears that zinc ions bind through sulphur and magnesium ions bind through oxygen (Table 3).

Discussion

Pyrophosphate analogues are of interest as antiviral compounds as they are product

inhibitors of polymerase/transcriptase enzymes and hence could inhibit viral nucleic acid synthesis without being metabolised. However, pyrophosphate is involved in many enzymic reactions in the host cells and hence pyrophosphate analogues may be cytotoxic as is the case with dichloromethylene bisphosphonate (3) (Cload, P.A. and Hutchinson, D.W., unpublished observations, 1983). Thus, it is significant that the mono- and bisthiopyrophosphates do not appear to be cytotoxic to MDCK cells after 36 h at concentrations at which they cause an appreciable reduction in the haemagglutinin titre of influenza virus A/X49. The thiopyrophosphates cause a 50% inhibition of the RNA transcriptase of this virus at concentrations similar to that for phosphonoformate (35 μM) and lower than that for inorganic pyrophosphate (125 μM).

We have suggested that the inhibitory effect on the RNA transcriptase of influenza virus A caused by pyrophosphate analogues is related to their ability to chelate zinc ions [3], and we believe that it is significant that both thiopyrophosphates are good chelators of zinc ions. The introduction of the 'soft' sulphur atom (on the HSAB scale) into the pyrophosphate residue might well alter the chelating properties of the molecule. It was, therefore, of interest to determine whether zinc ions coordinated to oxygen or sulphur in bisthiopyrophosphate. [^{31}P]NMR measurements (Table 3) show that when magnesium, zinc or cadmium ions were added to a solution of pyrophosphate in triethanolamine buffer at pH 8.2, the pH of the transcriptase assay, only small shifts (ca. 1 ppm) of the ^{31}P signal occurred, presumably due to the formation of complexes with the metal ions coordinated to the oxygen of the pyrophosphate.

In the case of bisthiopyrophosphate at pH 8.2, addition of a 'hard' metal ion (magnesium) produced a downfield shift of less than 2 p.p.m. On the other hand, addition of soft metal ions (zinc or cadmium) produced a large upfield shift of 8–9 ppm of the [^{31}P]NMR signal. Since cadmium ions have been shown to complex with sulphur in nucleoside thiophosphates [8], the [^{31}P]NMR results suggest that zinc ions probably coordinate to bisthiopyrophosphate through sulphur. If this method of coordination also occurs at the essential metal ion (probably zinc) in the transcriptase complex of influenza virus A, this may account for the difference in inhibitory activity between inorganic pyrophosphate and its thio-analogue.

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